FACTORS ASSOCIATED WITH CATION GRADIENTS IN THE MAMMALIAN ERYTHROCYTE

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Abstract

Mammalian erythrocytes have been shown to contain a potassium-activated enzyme, pyruvic phosphoferase (PPFase), and factors affecting its activity *in vivo* in relation to red cell sodium and potassium content have been studied. It has been shown that under certain conditions in laboratory animals an increase in the number of reticulocytes resulted in an increased activity of PPFase as well as an increased potassium content of the red cell.

Species activity of PPFase was found to be in the following decreasing order, man, rat, dog, rabbit, cat and ox. Chick erythrocytes and the immature cells of new born rats were found to possess very high activity, whereas the activity of cells from cord blood was only slightly above that of adult cells.

Clinical studies showed that during therapy of pernicious anemia, the PPFase activity and potassium content parallel the reticulocytosis, and that in general, the PPFase activity is increased in severe cases of anemia.

The synthesis of phospho(enol)pyruvate from phosphoglyceric acid has been shown with red cell hemolysates from human, rat and beef cells. In contrast to the PPFase, the phosphoglyceromutase-enolase system was found not to require potassium for maximum activity.

A method for choline acetylase, which showed the

presence of this enzyme in rat brain, failed to demonstrate any activity in erythrocytes. However it was found that the erythrocyte acetylcholinesterase, under certain conditions could synthesize acetylcholine from choline and sodium acetate by a reversal of its usual hydrolytic activity.

An *in vitro* investigation of nucleotide synthesis by red cells failed to show any evidence for potassium accumulation during synthesis. Loss of K^+ and gain of Na^+ in the presence of fluoride was observed. Phenylhydrazine *in vitro* did not affect the Na^+ or K^+ concentration of erythrocytes.

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GENERAL INTRODUCTION

Large concentration gradients of cations are exhibited by all living cells - usually high potassium inside and high sodium outside.

It has been known for many years that potassium "is of the cell and not the sap." However the controlling mechanisms responsible for such gradients are still a subject for speculation even today.

Since potassium is found in relatively large concentrations within most living cells, one may safely assume that it must affect cellular function and therefore vital processes and is, indeed, indispensable for life.

The importance of the potassium ion in physiological disturbances was recognized as early as 1878 (1), and its toxicity was recognized by Voit in 1868 (2). Death may result from hyperkalemia, or hypokalemia (2), (3). Alkalosis, acidosis, vomiting, diarrhea, surgery and fasting (4) are all known to affect potassium balance. The significance of the relation between potassium and the adrenal cortex, and carbohydrate metabolism, as well as its importance in the normal physiology of the nervous system, needs no comment.

Thus the realization of the profound influences resulting from a disturbed potassium metabolism has prompted research workers to investigate every possible

factor which may affect potassium balance in clinical medicine. Today, there are many comprehensive reviews on the subject (3), (4), (1).

Scientists realize however, that in order to be able to treat successfully a "deranged" potassium metabolism, the basic problem of what controls cellular ion accumulation must be solved first. Therefore we find that research on the problem has continued to grow. This began in 1940 with the Cold Spring Harbor Symposium on Permeability (5), and has rapidly expanded through the years as witnessed by such conferences as "Symposium on Metabolism of Potassium", which was held at the University of Minnesota, Sept. 1952.

"Permeability" of a membrane, in the strict sense of the term, - rate of flow of a substance across a membrane per unit time per unit area - can be measured quantitatively only by means of radioactive isotopes. For example, experimental data may show a constant intra-extra-cellular cation content and therefore indicate the conclusion that the membrane is "impermeable" to cations. Radioactive studies may show that the rates of penetration and loss are equal, thereby maintaining the system at a constant ionic composition. The obvious conclusion now becomes that the process is one of cationic exchange. It is not surprising to find that in the

literature the term "permeability" has become ambiguous and may include various meanings such as, penetration, cationic exchange, metabolic and physical processes occurring on the cell surface.

A comprehensive review of all the theories advanced for the mechanism of maintaining cationic gradients by the cell, as well as the arguments for and against such theories, would require several volumes. The writer, therefore, will mention only the more outstanding theories, and indicate the salient trends over the years.

The old belief that a cell's "permeability" is governed largely by the chemical composition and physical properties of its membrane, is found to be prevalent even in modern times. Early investigators therefore concentrated on the "permeability" or "impermeability" of the corpuscular membrane, assuming relatively inert and static conditions for the cell surface. Present day concepts of the membrane (10), (11), (12) picture the cell surface as consisting of lipoprotein complexes, with the lipids in a bimolecular layer at the surface, and a layer of protein micelles tangentially arranged beneath the layer of lipids. Altman (82) has shown that the erythrocyte surface is far from static; there is a rapid turnover of C^{14} constituents of the stroma.

One of the earliest investigators, Gürber 1895 (6), failed to find any exchange of electrolytes between the

blood cells and the plasma. Hamburger in 1916 (6), rediscovered the Nasse effect of 1878, that when red cells are exposed to carbon dioxide, they lose bicarbonate and gain chloride. This is the well known chloride shift and is still sometimes referred to as the "Hamburger phenomenon".

Overton's lipid solubility theory of 1895, although not dealing directly with inorganic ions, is important for the fact that it directed research on cellular "permeability". His theory is still used today to explain the actions of narcotics.

Even as late as 1940, statements in textbooks (7) can be found to the effect that "sodium and potassium cannot pass from the red cell into the plasma, because the red cell membrane is impermeable to cations with the exception of the hydrogen ion". The potassium within the cell was regarded as having been accumulated during the course of its maturation.

Passive ion accumulation is argued against by the very high electrical resistance of the membrane. Krogh (13) refers to Collander's work on plants (Characeae) where it was shown that the passive "permeability" to ions is of a very low order.

A "molecular sieve" or "charged pore" mechanism is indicated by Parpart's observation (5) that calcium ions inhibit the well known rapid exchange of anions in the red blood cell. That is, the calcium ion must have

reacted with certain lipid groups in the membrane thereby altering the pore size or charge.

Lundegärdh (14), (13) is considered to be one of the first to indicate a mechanism for active ion transport. He suggested that the cell surface is a mosaic structure of positive and negative groups with negative groups predominating. Such negative (strongly acidic) and positive (weakly basic) groups could act as "carriers" for ions. These carriers are capable of swinging or turning round in the membrane thus enabling them to exchange the attached ions with the interior of the cell. On the other hand, Conway showed that fermenting yeast (15) produced hydrogen ions which exchanged directly (no "carriers" necessary) with the potassium ions of the medium at the cell surface. From such studies he postulated a "redox pump" mechanism at the cell interface (27). That the process may require two different mechanisms is indicated by Krogh (13). He believed that a "steady state" of a fully grown cell is maintained by an active uptake to replace that lost by passive diffusion. Ussing (17) also proposes a double factor system in his theory of "exchange diffusion". This requires a carrier system in or on the surface which could complex with ions, and another system inside which could react with the complex to free the ion.

With the advent of the use of radioactive elements in experimental biology (19), coupled with the later introduction of the highly refined technique of sodium and potassium analysis by flame photometry, many concepts concerning cation "permeability" have had to be revised. Consequently we find that research in this field has shifted from emphasis on the membrane, to metabolic studies of the cell. It is now believed that a more active, dynamic type of regulation must control cation gradients with the required energy being derived from glycolysis.

Hevesy and Hahn (19), using radioactive potassium and sodium, were the first to show that the red cell membrane was "permeable" to both. Thus the old "impermeability" theory could no longer account for the differences in cation concentration between the cell and extra-cellular fluid. The studies of Weller and Taylor (20) revealed that potassium is constantly being exchanged between the plasma and the red blood cell and that all of the cellular potassium is exchangeable.

Animal experiments (3) with K^{42} showed that the cellular membranes are extremely permeable to potassium, the degree varying with the metabolic activity of the cell.

Maizels (23) confirmed Harris' observation (36)

that blood stored in the cold lost potassium and gained sodium and that this was reversible on incubation at 37°C. Further, this reversal was activated by glycolysis. Maizels therefore postulated that the primary activity in cation regulation is a sodium pumping mechanism, and that potassium accumulation is passive and secondary to the sodium extrusion. This is similar to the theory of the sodium pump in nervous activity advanced by Hodgkin (18).

Other workers (24) also provided evidence that the necessary energy required for the transfer of potassium was provided by the metabolism of glucose. The temperature coefficient, rate of transfer of potassium, and the rate of utilization of glucose by the red cell bore a direct relationship to one another.

Well-known glycolytic inhibitors have been shown to cause loss of potassium (30), and Mudge (35) demonstrated that rabbit kidney slices depleted of potassium can accumulate the cation in direct relation to oxidative phosphorylation. Studies of an indirect nature provide further evidence of the importance of carbohydrate metabolism in the transfer of potassium across the cellular membrane. It has been observed (8) that, in diabetic acidosis, changes in the organic acid-soluble phosphorus and potassium in the blood cells are closely parallel. Also, glycogen synthesis from glucose in

rabbit liver slices has been shown to be potassium-dependent (26).

One of the most attractive theories for potassium accumulation was advanced by Greig (28a-g) in which she related the cholinesterase-choline-acetylase system of the red blood cell to the cation-controlling mechanism. She showed that dog cells suspended in bicarbonate or in presence of cholinesterase inhibitors, lost potassium, while in the presence of acetylcholine but not benzoylcholine, potassium was replaced. Furthermore, she reported acetylcholine synthesis by acetone powder extracts of human blood cells (28h). However there have been numerous objections to her theory, the most convincing one of which was the demonstration by Mathias and Sheppard (29), that the ability of the red cell to synthesize acetylcholine was very doubtful. If this synthesis did occur, the rate would be very much lower than that required to provide the free energy essential for the accumulation of potassium against a concentration gradient.

Recently, Boyer (33) has indicated a number of potassium-activated enzymes. Muscle pyruvic phosphoferase, while activated by potassium, was shown to be inhibited by sodium (32). Melchior (34), believes that it is through complex formation with adenosinetriphosphate that sodium and potassium may exert their effect on enzymatic reactions.

Such a system may also account for the selective transfer of potassium. That is, the potassium-adenosine-triphosphate complex formed during metabolism may be utilized by an adenosine-triphosphate-requiring, potassium-activated enzyme.

In summary, one may conclude that the classic point of view, that the cation gradient in cells is essentially due to the permeability properties of the membrane, is no longer tenable. We know that potassium is not fixed intracellularly, but is completely exchangeable; it can move in the body rather freely according to the demands of shifting membrane equilibria; its concentration intracellularly is affected by acid-base balance, hormones, and carbohydrate metabolism. We also know that there is a very close relationship between potassium metabolism and glycolysis, with glycolysis providing the phosphorylated intermediates which are responsible for providing the energy for potassium accumulation. That is, the emphasis now is on the importance of a continuous supply of energy for the maintenance of the cationic gradients in living cells.

However it appears that even this conclusion is oversimplified. Sheppard and Stewart (31) have found that while radiations will cause potassium loss from blood cells, there is no effect on the glycolytic mechanism,

ATP production, cholinesterase activity, or the red cell membrane as evidenced by a lack of hemolysis. This is in sharp contrast to the previous recorded statements where potassium loss is correlated with inhibition of glycolysis.

Thus we see that the problems concerning specific ion transport mechanisms are indeed many and complex. The answer in all probability will not lie in any simple and clear cut process, but rather in a combination of several processes acting together.

It was believed that a better understanding of the problem would be obtained if a correlation could be shown between the activity of a potassium-activated enzyme, and the intracellular concentration of potassium.

This thesis reports on factors affecting the cation gradients in the red cell in vivo and in vitro. It also reports the occurrence in the red corpuscles of a potassium-activated enzyme, its properties, and factors affecting its activity in relation to cation gradients. A study of the cholinesterase-choline-acetylase system, as well as the synthesis of pyridine nucleotides and the effect on cation concentration is reported. Furthermore, this thesis indicates the presence in the erythrocytes of a phospho-glycero-mutase-enolase system which can form phospho(enol) pyruvic acid from phosphoglyceric acid. This system was shown to be K^+ independent in contrast to pyruvic phosphoferase which is K^+ dependent.

EXPERIMENTAL

IA. Pyruvic Phosphoferase Activity Of The Red Blood Cell

1. Introduction

Today it is realized that in order to gain an accurate understanding of the controlling mechanisms regulating cation gradients, the complex cellular enzymatic systems must be studied individually.

Both Ariel (4) and Elkinton (21), from studies on clinical potassium disturbances, point out that tissue sodium and potassium concentrations may vary independently of each other. They found that a potassium loss may be followed either by an increase or a decrease of sodium, or that an increase in sodium may occur without a change in potassium.

Scott and Hayward (22), from studies on cation distribution in green algae following various glycolytic and photosynthetic inhibitors, came to a similar conclusion. That is, the two ions are under separate metabolic control. They suggested that the energy for the potassium "pump" came from below the level of the phosphoglycerate, while the sodium "pump" was geared to the metabolism of pyruvate or a product of its metabolic degradation.

Recently, Boyer (32) has shown that the potassium ion activates, while sodium inhibits, the muscle enzyme

pyruvic phosphoferase. He also listed other enzymes which are activated by potassium, such as, phospho-fructokinase, fructokinase, choline acetylase, malic enzyme, phosphotransacetylase, aldehyde dehydrogenase and lactase (33).

Melchior (34), has indicated a possible mechanism for such ion antagonism. He demonstrated that the size of the cation determined the shape of the adenosinetri-phosphate (ATP) complex which it found, and that the specific shape of such a complex may determine the rate of enzymatic reactions. Such a system may also account for the selective transfer of potassium. For example, the potassium adenosinetriphosphate (K-ATP) complex which is formed during cellular metabolism, may be utilized by an ATP-requiring, potassium-activated enzyme, such as fructokinase or choline acetylase.

The demonstration for the first time of a specific activation by potassium of an enzyme reaction, was for the muscle enzyme, pyruvic phosphoferase* (32).

This enzyme is known to play an essential role in the glycolytic process, transferring the energy-rich phosphate of phospho(enol) pyruvic acid, to

* The following abbreviations and symbols will be used: PEPA, phospho(enol) pyruvate; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; PPFase, pyruvic phosphoferase; TRIS, tris(hydroxymethyl) aminomethane-hydrochloride; PCMB, p-chloro mercuribenzoate; Ach, acetyl-choline.

adenosine diphosphate according to the following equation:

$$PEPA + ADP \rightleftharpoons Pyruvate + ATP.$$

The absolute requirement for K^+, Rb^+, or NH_4^+, by this enzyme, was shown by Kachmar and Boyer (39). From a study of 19 different tissues in 11 vertebrate and invertebrate organisms with respect to PPFase activity, Boyer (40) concluded that PPFase activation by K, is a general phenomenon since it was activated in all cases. However, no blood cells have been studied.

Therefore it was of interest to see whether PPFase occurred in the red blood cell, and if so, whether factors affecting its activity had any effect on the potassium distribution.

2. Experimental

a. Procedure for Enzyme Assay

The method used for enzyme assay was that of Kachmar and Boyer (39), with slight modifications.

The blood samples were collected into heparinized tubes, washed* two times with 0.15 M KCl, and the buffy layer was carefully aspirated off each time.

The packed cells were diluted approximately ten times with isotonic KCl and aliquots taken for cell counts and enzyme determination. For human blood, 1.0 ml. of this

*that is, the erythrocytes were washed after the plasma was drawn off

1:10 cell suspension was centrifuged and the KCl very carefully aspirated off, after which 5.0 ml. of water was added to the cells. For each enzyme determination 0.10 ml. of such a hemolysate was used.

Assays which gave results over 0.40 μM/ml. were repeated with an appropriate enzyme dilution. This was especially important in testing for species activity and in anemias. For example, cells from one-day old rats had to be diluted a total of 1:200, instead of the 1:50 dilution as for adult human blood. Extreme care must be exercised not to cause hemolysis during the handling of the cells as the enzyme was found to be located intracellularly.

Phospho(enol) pyruvic acid was obtained as the Ag, Ba salt (California Foundation for Biochemical Research). The potassium salt was prepared by treatment with N HCl in an ice bath, followed by addition of 0.5 M K_2SO_4 until no more $BaSO_4$ precipitated. This was allowed to stand overnight, centrifuged, and carefully brought to the right pH with concentrated KOH. ADP was used as the sodium salt (Sigma Chemical Co.); it was found necessary to prepare solutions fresh daily in order to obtain correct blank readings.

The incubation mixture was made up to the following final molar concentrations: PEPA, 0.003; ADP, 0.004;

Tris buffer, 0.040, pH 7.4*, KCl, 0.10; $MgSO_4$, 0.005. This was made up to a total 0.9 ml., and after five minutes for thermo-equilibration, 0.10 ml. of hemolysate was added. The incubation took place for exactly 10 minutes at 37°C. Controls lacking only in ADP were run simultaneously and were used for the blank readings.

At the end of the incubation time, 1.0 ml. of 0.0125% 2,4-dinitrophenylhydrazine in N HCl was added directly to the 1.0 ml. of incubation mixture in the tubes. After mixing, this was allowed to incubate for another 10 minutes at 37°C. to allow the formation of the dinitrophenylhydrazone of pyruvic acid. Then 3.0 ml. of 0.5 N NaOH was added, (the hydrazone develops a red color in the presence of base), mixed, and after 5 minutes at room temperature, the precipitated $Mg(OH)_2$ and protein, were centrifuged off at 3000 r.p.m. for 5 minutes.

Under these conditions the pyruvate liberated was found to vary directly with enzyme concentration up to about 1.0 μM/ml. Units of PPFase activity were expressed as micromoles of pyruvate liberated in 10 minutes at 37°C., pH 7.4 in 1.0 ml. of incubation mixture. For comparative purposes, the activity was expressed as micromoles per 10^9 cells.

Photometric readings were made in the Beckman DU

* The optimal pH was found to be 7.0, however it is desirable to follow standard conditions already published in the literature for comparable results, and since the pH optimum has a wide range, it was decided to use pH 7.4.

spectrophotometer at 510 mμ. Crystalline sodium pyruvate for the standard curve (table 1) was prepared from pyruvic acid by the method of Robertson (41). One ml. of standard was taken for analysis, but the final total volume was 5 ml. as read in the photometer. Unless stated otherwise, human blood was used throughout the experiments.

Table 1

Photometer Readings on Pyruvate Standards

μM pyruvate/ml.	% T	Absorbancy
0.01	99.0	.0044
0.02	98.0	.0088
0.05	94.5	.0246
0.10	89.8	.0459
0.20	79.9	.0969
0.50	58.0	.2366
0.75	44.4	.353
1.0	34.7	.459

b. Properties of Enzyme

i. Effect of storage on enzyme activity:

To test the effect of storage on enzyme activity, blood was drawn with a sterilized syringe and immediately delivered in three ml. aliquots into a series of sterilized test tubes containing acid-citrate-dextrose solution (2 gm. disodium citrate monohydrate and 3 gm. anhydrous glucose made up to 100 ml.). The tubes were stored at 5°C. and assayed for enzyme activity on the days indicated.

Table 2a

Effect of Storage on Enzyme Activity

Days of Storage	Activity (μM/ml.)
nil	0.82
2	0.80
5	0.76
8	0.74
11	0.74
15	0.63
21	0.47
29	0.21

Table 2a indicates the enzyme is stable for at least two days in red cells when whole blood is stored in acid-citrate-dextrose solution at 5°C. There is only a slight decrease in enzyme activity from the second to the eleventh day with a marked decrease after the fifteenth day. About 75 percent of the enzyme activity is lost by the twenty-ninth day of storage.

ii. Dialysis of Hemolysate

In order to study the specific effects of various added ions it is important not to have any contaminating ions present, therefore it was decided to use dialysed hemolysates. Human red cell hemolysates were dialysed against Tris buffer pH 7.4, at 5°C. The albumin used was that of Armour Laboratories Ltd., crystalline bovine plasma albumin. Table 2b indicates the results of the dialysis studies.

Table 2b

Effect of Dialysis on Enzyme Activity

Human Red Cell Hemolysates		
Time of Dialysis	Dialysed in Presence of	Percent Loss of Activity
12-30 hours	No additions	70-90
30 hours	2% albumin	0-5
15 hours	2% albumin	nil
30 hours	0.02 M cysteine	39
Rat Muscle		Activity*
15 hours	No additions	0.30 μM/ml.
Nil	Control	0.30 μM/ml.

* Micromoles of pyruvate liberated in 10 minutes at 37°C.

In contrast to the rat muscle enzyme, red cell PPFase was inactivated on dialysis. However the presence of albumin afforded complete protection against such inactivation, while cysteine afforded only a partial protection (60%).

The possibility that the loss of activity was due to some dialyzable activator was ruled out by the addition of the dialysate, yeast extract, Ach. and other factors as summarized in table 2c. It can be seen from the table that no reactivation was obtained, and therefore it is concluded that the loss of activity is due to an inactivation of the enzyme on dialysis.

Table 2c

Re-activation of Dialysed Hemolysates

Compound tested	Conc. (M)	Time in contact with dialysed hemolysate prior to assay	Percent re-activation
Ach*	1×10^{-2}	nil	nil
DPN[2]	few crystals	nil	nil
TPN[3]	few crystals	nil	nil
GSH[4]	1×10^{-2}	nil	nil
Cysteine	2×10^{-2}	24 hours	nil
Vitamin B12	few crystals	nil	nil
Boiled yeast extract		nil	nil
Boiled hemolysate from day-old rats		nil	nil
Dialysate		nil	nil
Same dialysate but conc. four times (under 28" of vacuum and 35°C.)		4 hours	nil

*Acetylcholine [2]Diphosphopyridine nucleotide

iii. Effect of Acetone Extraction on Enzyme Activity

The erythrocytes were washed two times with 1% NaCl and ground in cold acetone (8°C.) for 15 minutes. An aqueous extract of the acetone powder was made and the supernatant assayed for activity.

Table 2d

Effect of Acetone Extraction on Enzyme Activity

	% T	Activity (μM/ml.)
Acetone extract	72.0	0.31

Therefore it can be seen that acetone extraction does not inactivate the enzyme.

[3] Triphosphopyridine nucleotide [4]Glutathione

iv. Location of Enzyme in the Erythrocyte:

One ml. of packed cells was extracted successively five times with an equal volume of water. The red cell stroma was centrifuged out each time at 12,000 r.p.m. for 10 minutes. Percent enzyme activity, is based on the original hemolysate (minus stroma) as 100 percent.

Table 3

Location of Pyruvic Phosphoferase In The Erythrocyte

Extract Number	Activity (%)
Original hemolysate (minus stroma)	100
1	12
2	5
3	1
4	0
5	0
The extracted stroma suspension	0

From the above results it may be concluded that the enzyme is located entirely intracellularly.

c. Kinetics

For the kinetic studies, non dialysed hemolysates were used and the following factors as affecting enzyme activity were studied; the effect of, the time of incubation, the effect of enzyme concentration, the effect of pH, the effect of substrate concentration, and the effect of ADP concentration. These results are summarized in tables 4, 5, 6 and 7 and 8.

Table 4

The Effect of the Time of Incubation on Enzyme Activity

Time (minutes)	Activity (μM/ml.)
3	0.10
6	0.20
9	0.30
12	0.40
20	0.60

Table 5

Effect of Enzyme Concentration on Enzyme Activity

Ml. of enzyme	Activity (μM/ml.)
0.025	0.19
0.05	0.39
0.10	0.79
0.15	1.13

Table 6

Effect of pH on Enzyme Activity

pH	Activity (μM/ml.)
6.3	0.24
6.6	0.30
7.0	0.34
7.4	0.30
7.7	0.23

Table 7

Effect of Substrate Concentration on Enzyme Activity

PEPA final conc. (M)	Activity (μM/ml.)
0.004	0.30
0.003	0.32
0.002	0.30
0.001	0.28

Table 8

Effect of ADP* concentration on Enzyme Activity

ADP (final conc.) M	Activity (μM/ml.)
0.008	0.33
0.006	0.40
0.004	0.41
0.002	0.33

* Adenosinediphosphate

Under the experimental conditions used the erythrocyte PPFase was found to have a wide range of optimal pH, 6.6 - 7.4, with the optimum at pH 7.0. The optimal final molar concentrations of the various compounds used were as follows: PEPA, 0.003; ADP, 0.004 - 0.006; KCl, 0.05 - 0.15; $MgSO_4$, 0.005 - 0.010. Under these conditions the pyruvate liberated was found to vary directly with enzyme concentration up to 1.0 μM/ml.

d. Ion Effects

Dialysed hemolysates were used in all cases of monovalent cation studies with the exception of Ag^+. The results of all the ion studies are summarized in tables 9 - 16 and are discussed on page 31.

Ion Effects

Table 9

Optimal Mg^{++} Requirements*

Mg^{++} Conc. (final) M.	Activity (μM/ml.)
1×10^{-1}	0.06
1×10^{-2}	0.44
5×10^{-3}	0.44
1×10^{-3}	0.25
nil	0.00

*Non dialyzed hemolysate

To find the optimal K requirements the hemolysate was dialysed for 15 hours in the presence of 2% albumin.

Table 10

Optimal K^{+} Requirements

K^{+} Conc. (final) M.	Activity (μM/ml.)
0.15	0.215
0.100	0.220
0.050	0.215
0.010	0.155
0.001	0.07
nil	0.00

Table 11

Ability of Monovalent Ions to Replace K^+
(i.e. in the absence of K^+)

Ion	Conc.(final) M.	Activity (μM/ml.)	Max. Activation*
Li^+	1×10^{-1}	0.00	0.00
Rb^+	1×10^{-1}	0.14	0.87
NH_4^+	1×10^{-1}	0.142	0.88
K^+	1×10^{-1}	0.16	1.0
Ach^+	1×10^{-1}	0.00	0.00

For the above experiment (table 11) the hemolysate was dialysed for 15 hours in the presence of 2% albumin.

* Maximum activation in the absence of K^+, giving K^+ an activity of 1.0.

Table 12

Effect of Na^+ and Ca^{++} in the Presence of Both Mg^{++} and K^+

Ion	Conc.(final) M.	Activity (μM/ml.)	% Inhibition
Na^+	1×10^{-1}	0.22	16.
	1×10^{-3}	0.26	0
	0	0.26 (control)	0
Ca^{++}	1×10^{-1}	0.00	100
	1×10^{-2}	0.09	86
	1×10^{-3}	0.46	30
	1×10^{-4}	0.66	0
	0	0.66 (control)	0

Table 13

Effect of Increasing K^+ Concentration on Ca^{++} Inhibition

In Presence of K^+ Only			Additional Presence of 10^{-3} M Ca^{++}		
Conc.(M)	Activity (μM/ml.)	% Inhibition	μM/ml.	% Inhibition	"Net" Ca^{++} % Inhibition
1×10^{-1}	0.66	Control	0.41	38	38
2×10^{-1}	0.55	17	0.37	44	27
4×10^{-1}	0.32	52	0.25	62	10

Table 14

Ability of Divalent Ions to Replace Mg^{++}
(i.e. in the absence of Mg^{++})

Ion	Conc.(M) final	Activity (μM/ml.)	Max. Activation
ZN^{++}	5×10^{-3}	0.00	0.00
Ni^{++}	5×10^{-3}	0.00	0.00
Sr^{++}	5×10^{-3}	0.00	0.00
Co^{++}	5×10^{-3}	0.14	0.24
Mn^{++}	5×10^{-3}	0.28	0.48
Mg^{++}	5×10^{-3}	0.59	1.00

Table 15

Effects of Heavy Metals on Enzyme Activity

Ion	Conc.(M) final	Activity(μM/ml.)	% Inhibition
Control		0.45	
Pb^{++}	1×10^{-4}	0.31	30
$UO_2(NO_3)_2$	1×10^{-4}	0.18	60
ZN^{++}	1×10^{-4}	0.12	74
	1×10^{-3}	0.00	100
Ag^{+}	1×10^{-4}	0.02	96
Control		0.20	
Cu^{++}	1×10^{-5}	0.18	10
	1×10^{-4}	0.04	80
	1×10^{-3}	0.00	100
Control		0.59	
Hg^{++}	1×10^{-5}	0.24	60
	1×10^{-4}	0.04	94
Ni^{++}	1×10^{-3}	0.41	30
	5×10^{-3}	0.08	87
Sr^{++}	5×10^{-3}	0.30	49
Co^{++}	5×10^{-3}	0.14	76
Mn^{++}	5×10^{-3}	0.27	53

Table 16

Effect of Increasing Mg^{++} Concentration on Mn^{++} AND Co^{++} Inhibition

Mg^{++} Conc.(M)	Co^{++} or Mn^{++} Conc. (Molar)	Activity (μM/ml.)	% Inhibition
5×10^{-3}	Control	0.59	
5×10^{-3}	5×10^{-3} Co^{++}	0.14	76
1×10^{-2}	5×10^{-3} Co^{++}	0.17	71
5×10^{-3}	5×10^{-3} Mn^{++}	0.28	52
1×10^{-2}	5×10^{-3} Mn^{++}	0.31	47

e. Effects of Various Inhibitors

Inhibition studies were carried out on undialyzed human hemolysates, and the results indicated in tables 17, 18, 19 and 20. A detailed discussion of the results is given on page 31.

Table 17

Effect of Various Inhibitors on Enzyme Activity

Inhibitor	Conc. (M)	Time of Incubation With Enzyme Prior to Assay	Activity (μM/ml.)	% Inhibition
Phenothiazone	1.2×10^{-4}	nil	0.79	5
	3×10^{-5}	nil	0.63	9
Control			0.83	
Phenothiazine	1×10^{-3}	1.25 hours	0.30	nil
5% ETOH (Control)		1.25 hours	0.30	
Urethane	1×10^{-2}	nil	0.30	nil
Alloxan	5×10^{-3}	nil	0.30	nil
Control			0.30	
2,4-Dinitrophenol	1×10^{-3}	nil	0.59	9
Control			0.65	
Na-iodoacetate	1×10^{-3}	nil	0.72	nil
	1×10^{-3}	20 hours	0.52	28
Control			0.72	
Na-arsenate	1.6×10^{-2}	nil	0.28	6
Control			0.30	
K-ferricyanide	1×10^{-3}	nil	0.16	nil
	1×10^{-3}	1 hour	0.16	nil
	1×10^{-3}	6 hours	0.14	13
Control			0.16	
Na-fluoride	1×10^{-2}	nil	0.30	nil
Control			0.30	
Cortisone-Ac.	1×10^{-3}	nil	0.50	19
	1×10^{-4}	nil	0.58	7
Control			0.62	

Phenylhydrazine:

The in-vitro effect of phenylhydrazine on enzyme activity could not be determined due to its combination with the liberated pyruvic acid. For example, incubating a 10^{-3}M concentration of phenylhydrazine with a standard sodium pyruvate solution of 0.50 μM/ml., at pH 7.4 for 10 minutes, resulted in a loss (combination) of 75% of the added pyruvate.

Table 18

Effect of PCMB on Enzyme Activity

Time of Incubation with enzyme prior to assay (minutes)	Activity (μm/ml.) In Presence of 1×10^{-4}M PCMB
10	0
5	0
3	0
1	0
0	0

Table 19

Effect of 1×10^{-1} M. GSH on Re-activation of 1×10^{-4} M. PCMB Inhibition

Time of Inhibition	Time of Re-activation	Activity (μM/ml.)	Percent re-activation
1 minute	10 minutes	0.06	18
1 minute	15 minutes	0.15	47
1 minute	25 minutes	0.19	59
10 minutes	25 minutes	0.09	28
No inhibition	Control	0.30	

Table 20

Effect of 'SH' Compounds on Ion Inhibition

Ion	Conc.(M)	Inhibition				'SH' Compound Added Concentration (M)
		In absence of 'SH' Compound		In Presence of 'SH' Compound		
		Percent	μM/ml.	Percent	μM/ml.	
Hg^{++}	1×10^{-4}	94	0.04	57	0.26	Cysteine, 1×10^{-3}
	Control		0.59			
ZN^{++}	1×10^{-4}	73	0.12	nil	0.45	Cysteine, 1×10^{-3}
	Control		0.45			
Cu^{++}	1×10^{-3}	100	nil	100	nil	GSH, 2×10^{-3}
	1×10^{-4}	100	nil	nil	0.43	GSH, 1×10^{-2}

3. Discussion

The results shown in table 3, indicate that the enzyme is intra-cellular, therefore all the experiments were carried out using whole hemolysates. Furthermore, although data in table 8 shows that the enzyme is stable for at least two days, fresh blood samples were always used for the experiments. It was also found that acetone extraction does not inactivate the enzyme. Dialysis however, resulted in marked loss of activity which could be prevented by the addition of crystalline albumin prior to dialysis. This is in sharp contrast to the muscle enzyme which retained its complete activity on dialysis (table 2b). Kachmar and Boyer (39) used dialysed rabbit muscle extracts in their experiments, but made no mention of any inactivation of the enzyme due to dialysis. Therefore it would appear that this is the only property in which PPFase differs from the two different sources.

Kachmar and Boyer's observations on the effects of Na^+, Li^+, K^+, NH_4^+, Rb^+, Mg^{++} and Ca^{++} on PPFase activity were confirmed here. Both K^+ and Mg^{++} are required for enzyme activity. Ammonium and Rb^+, but not Li^+, could replace K^+. Sodium exhibited some inhibition in the presence of K^+ and Mg^{++}, while Ca^{++} caused marked inhibition which could be partially reversed by increasing the K^+ concentration. No other ion

studies were reported by Kachmar and Boyer (39).

Table 14 indicates that both Mn^{++} and Co^{++} may partially replace Mg^{++}, but the Ach^{+} cation could not replace K^{+} (table 11). It is interesting to note that the enzyme phosphoglucomutase is also activated by Mn^{++}, Co^{++}, and Ni^{++} as well as Mg^{++} (43). Both Ni^{++} and Sr^{++} had inhibitory effects and could not replace Mg^{++} in PPFase activity. However Co^{++} and Mn^{++} exhibited inhibitory effects in the presence of Mg^{++} which appeared to be of a competitive nature (table 16).

Inhibition experiments with heavy metals and PCMB have led to the conclusion that this is an 'SH' enzyme. This confirms Needham's observation (37) that pyruvic phosphoferase is an 'SH' enzyme. Barron (42), states that "possibly all enzymes dealing with adenosinetriphosphate transfer reactions, have been shown to be 'SH' enzymes."

Copper, ZN^{++}, Ag^{+} and Hg^{++} were found to be the most potent inhibitors, while the presence of glutathione (GSH) or cysteine generally resulted in complete protection from such inhibition. Table 19 illustrates the re-activation by GSH of PCMB inhibition.

Thiazine derivatives and other organic compounds such as urethane, 2,4-dinitrophenol, and iodocetate exhibited no appreciable inhibition (table 17).

B. Pyruvic Phosphoferase Activity Of The Erythrocyte In Various Species, In Immature Cells, And In Nucleated Cells.

1. Introduction

It is well known that the red blood cells of certain species, such as cat, dog, ox, and goat contain a low K and high Na concentration (44, p. 121). In contrast, the erythrocytes of man, rabbit, rat, pig and birds (19, p. 484) contain a high K and low Na concentration.

A study of the PPFase activity of the erythrocytes in the different species should, therefore, indicate whether there is any relationship between a potassium-activated enzyme and the intracellular concentration of potassium.

2. Experimental

a. Procedure

1. Enzyme determination:

PPFase activity was determined according to the procedure outlined on page 13.

In order to keep the enzyme activity around 0.4 μM/ml. it was found necessary to make the following approximate total cell dilutions: adult human, 1:50; fetal human, 1:60; adult rat, 1:50; fetal rat, 1:200; chick, 1:200; ox, 1:25;

cat and dog, 1:20; rabbit, 1:40. Fresh blood samples were used in all cases.

ii. Reticulocyte counts:

Reticulocytes were stained with the following formula (45):

New Methylene Blue, Color Index No. 927	0.5 gm.%
Potassium oxalate	0.65 gm.%
Distilled water	to 100 ml.

Store in the cold (5°C.).

Equal volumes of blood and formula (leaving an air bubble in between the two liquids) are drawn up into a capillary tube, blown out onto a glass slide, mixed by alternate suction and blowing and finally left in the capillary for 10-20 minutes. A blood smear is then made in the usual manner and 1000 cells counted under oil immersion.

3. Results

Table 21

Mean Erythrocyte Phosphoferase of Mammalian Species

Species	No.	MCV* (μ^3)	PPFase Activity		
			μM/10^9 cells		μM/ml. (Mean)
			Range	Mean ± S.E.	
Man	16	87	8.0 - 15.2	11.3 + 0.52	130
Rat	18	61	4.0 - 10.6	7.2 + 0.51	118
Dog	10	66	4.1 - 11.0	5.9 + 0.70	89
Rabbit	9	61	3.3 - 7.4	4.8 + 0.50	79
Cat	8	57	1.9 - 4.7	3.4 + 0.32	60
Ox	7	48	1.4 - 2.8	2.1 + 0.17	44

* Taken from Standard Values in Blood, E. C. Albritton, ed., American Institute of Biological Sciences, Washington, D. C., 1951.

Table 22

Mean Phosphoferase of Nucleated and Immature Red Blood Cells

Species	Age	No.	PPFase Activity, μM/10^9 cells		Retics %
			Range	Mean ± S.E.	
Chick	1 month	5	75 - 107	87 + 5.2	
Human	Cord blood	10	10.0 - 21.3	17.1 + 1.67	4
Rat*	fetal	1		95	100
	1-day	3	88 - 124	102	98
	3-days	1		110	78

* The blood from 5-8 young rats of the same litter was collected by decapitation and pooled for the PPFase determination. The values in the 3rd column represent the number of such pools analysed.

To determine whether any PPFase activity was associated with the nuclei of chick cells, one hemolysate was divided into two parts. Part A was centrifuged at 12,000 r.p.m. for 15 minutes, while part B was not centrifuged. Table 23 indicates the results.

Table 23

Phosphoferase Activity of the Nuclei of Chick Cells

Procedure	Part	Activity (μM/ml.)
Centrifuged	A	0.69
Not centrifuged	B	0.70

4. Discussion

Under the experimental conditions used, the PPFase activity of the erythrocytes of the dog, cat, and ox exhibited a lower activity than that of man, rat and bird. However it is well to remember that the optimal K^+(0.10 M) and Mg^{++}(0.005 M) concentrations were determined for the human enzyme only, and these may not necessarily be the optimum concentrations for the PPFase of all the species. Until this problem has been investigated, no definite conclusion may be drawn as to a possible relationship between the low enzyme activity and the low K^+ concentration of these cells.

Table 22 shows that a very high PPFase activity is associated with chick cells, and with immature erythrocytes of the rat. The extremely high PPFase activity of the cells of immature rats, appears to be associated with the increased number of reticulocytes. Nucleated red cells are known to have a very high aerobic metabolism, but their glycolytic activity is quite low (46). Centrifugation of hemolysates of the chick cells showed that the PPFase activity is not associated with the nuclei (table 23).

Jones and McCance (47) found that several enzymes of human cord blood, including cholinesterase, catalase and glyoxalase were less active than in adult blood, however table 22 indicates that red cells from human cord blood have a higher mean PPFase activity than those of adults.

C. Pyruvic Phosphoferase Activity Of The Mammalian Erythrocyte In Various Experimental States Of Anemia, and in Tumour bearing rats.

1. Introduction

Previous experiments (see section IB) have indicated that an increased PPFase activity is associated with an increased number of reticulocytes. Therefore it was of interest to follow the PPFase activity in experimentally induced anemia in laboratory animals.

2. Experimental

a. Procedure

i. Enzyme determination:

PPFase activity was determined according to the procedure outlined in section IA. Heparinized tubes were used throughout the experiments for collecting blood samples.

ii. Production of Anemia:

Anemia in rabbits was produced by injecting acetylphenylhydrazine (AcPHZ) subcutaneously as a 1% solution in warm physiological saline at 20 mg./Kg. body weight. Anemia was also produced by blood loss in both rats and rabbits by means of cardiac puncture . Rats were anesthetized with ether in a desiccator.

For cardiac puncture in rats, best results were obtained

by using a short 24 gauge needle. If larger size needles (21 gauge) were used, or if the needle had to be inserted several times, the rats invariably died from internal hemorrhages due to needle lacerations. Therefore if the heart was missed on the first occasion, the rat was put back in its cage, and a second attempt was made an hour or two later.

Where vitamin B_{12} (Merck's Cobione, 15 gamma/ml. in saline) was used, it was administered intramuscularly.

iii. Sodium and Potassium Determination:

The cell sodium and potassium determinations were done in a Beckman DU quartz spectrophotometer with flame attachment. The determinations were carried out immediately after blood withdrawal according to the following procedure.

Micro-hematocrits were done in duplicate, with specially designed commercial capillary tubes. They were centrifuged at 3000 r.p.m. for 30 minutes in all cases. For the cation analysis, one-half ml. of whole blood was washed two times with about 12 ml. of 0.3M sucrose. The centrifuging was again standardized to 5 min. at 3000 r.p.m. in all cases. After the sucrose was carefully aspirated off, the cells were diluted 1:200 with water and sprayed through the burner. A standard containing 10 meq./L of Na^+ and 100 meq./L of K^+, was sprayed through the burner immediately before and after each sample.

Final sodium and potassium concentrations per liter of packed cells were calculated on the basis of the micro-hematocrit values.

All glassware used in any way for the sodium and potassium determinations was washed with a special nitric-sulfuric acid cleaning solution. Also due care was exercised during the handling of the samples while in the 5 ml. "spraying" beakers in order to avoid Na^+ contamination from the fingers.

3. Results

In general, the anemia following acetylphenylhydrazine injections in rabbits was followed by a marked reticulocytosis. The results of such studies as well as those of the rat experiments are summarized in the following tables and discussed on page 50.

a. Rabbit Experiments

Table 24

The Effect of a Single Injection of AcPHZ on the PPFase Activity of the Erythrocytes (Rabbit No. 1)

Days After Injection	RBC x 10^6	MCV(μ^3)	Enzyme units
Prior to injection	5.64	72	3.4
2	4.37	62	1.3
3	3.00	71	3.9
4	2.34	-	5.6
5	1.18	85	11.3
6	1.42	137	30.0
7	1.46	133	36.0
9	2.00	113	34.0
11	2.64	112	24.0
14	3.77	100	13.5
18	4.72	85	8.0
25	5.28	76	8.6
32	5.40	69	6.2

Table 25

The Effect of a Single Injection of AcPHZ on the PPFase Activity and Cation Distribution in the Erythrocytes
(Rabbit No. 2, two experiments)

Days after Injection	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	meq./L packed cells		
					K^+	Na^+	Total base
Prior to injection	5.48	68	2	5.6	94.6	9.0	103.6
1	4.74	79	-	6.8	68.6	14.9	83.5
2	3.80	59	6	3.5	59.4	23.7	83.1
3	2.48	67	18	6.9	63.5	25.7	89.2
5	1.40	97	58	8.2	97.8	14.0	112.0
7	1.48	132	70	2.0	97.0	5.0	102.0
8	rabbit died						

Table 26

The Effect of Repeated Injections of AcPHZ on the PPFase Activity and Cation Distribution in the Erythrocytes
(Rabbit No. 4, one experiment)

Date and amount of injection	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	meq./L packed cells		
					K^+	Na^+	Total base
May 31 - 45 mg.	6.85	59	3	3.3	92.0	11.1	103.1
June 2 - 30 mg.	5.30	61	5	2.8	83.0	14.4	97.4
June 4 - nil	2.70	71	25	1.0	77.6	20.7	98.0
June 7 - 25 mg.	2.10	111	40	9.0	101.0	8.5	109.5
June 9 - 30 mg.	2.72	112	55	0.0	101.0	13.3	114.3
June 11 - nil	3.10	89	60	4.6	103.0	9.0	112.0
June 15 - 35 mg.	3.60	93	80	0.5	100.0	11.6	111.6
June 17 - 40 mg.	3.00	85	95	3.0	112.0	15.4	127.4

Table 27

The Effect of A Single Injection of AcPHZ plus Vitamin B12 on the PPFase Activity and Cation Distribution in the Erythrocytes
(Rabbit No. 5, one experiment)

Date and amount of Vitamin B12 Administration	Date of Assay	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	meq./L packed cells		
						K^+	Na^+	Total base
May 31 - 1.5 γ	May 31	5.55	70	4	7.4	96.3	9.5	105.8
June 2 - 1.5 γ	June 2	4.30	69	6	2.7	100.0	13.7	113.7
June 4 - 1.5 γ	June 4	3.20	72	18	4.5	93.4	10.0	103.4
June 7 - 1.5 γ June 8 - 3.0 γ June 9 - 3.0 γ	June 7	2.55	111	25	3.0	95.6	6.2	101.8
	June 9	3.70	99	17	7.0	105.0	5.0	110.0
	June 11	4.48	88	11	0.0	103.0	5.5	108.5
	June 15	4.55	93	4	5.2	103.0	7.8	111.0
	June 17	5.10	82	3	2.1	105.0	5.8	110.8

Table 28a

The Effect of Repeated Blood Loss plus Vitamin B12 on the PPFase Activity and Cation Distribution in the Erythrocytes
(Rabbit No. 6, one experiment*)

Date and amount of blood taken	Amount of Vit. B12 Administration	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	meq./L packed cells		
						K^+	Na^+	Total base
May 31 - 12 ml.	1.5 γ	6.55	69	3.0	5.1	93.3	11.8	105.1
June 2 - 2 ml.	1.5 γ	5.65	70	3.4	2.0	97.0	11.0	108.0
June 4 - 14 ml.	1.5 γ	5.50	77	5.0	2.2	100.0	12.5	112.5
June 7 - 20 ml.	3.0 γ	5.50	67	4.0	4.5	101.0	14.2	115.2
June 8 - 11 ml.	3.0 γ							
June 9 - 40 ml.	3.0 γ	4.55	69	10.0	6.0	103.0	15.6	118.6
June 10 - 33 ml.	4.5 γ							
June 11 - 13 ml.	3.0 γ	3.00	77	14.0	8.5	106.0	13.8	119.8
June 12 - 40 ml.	6.0 γ							
June 15		3.90	84	40.0	2.0	102.0	12.4	114.4
June 17		5.20	66	10.0	0.2	102.0	10.4	112.4

* A control rabbit which received equivalent saline injections instead of the vitamin B12, gave results identical with those in the above table.

Table 28b
Cation Content of Rabbit Erythrocytes
Average Values For 6 Normal
Rabbits in This Laboratory

Meq./L cells	Mean	Range
K^+	95.0	91.6 - 102.0
Na^+	10.0	9.0 - 11.8

b. Rat Experiments

Eight male rats of approximately the same weight (285 gm.) were divided into two groups of four each. Each rat was bled 6 ml. by cardiac puncture on the first day of the experiment. Subsequently, only 2 ml. of blood from each rat was withdrawn on the designated date. Group A received daily injections of 0.062γ Vit. B_{12} in 0.1 ml. of saline. Group B received daily injections of 0.1 ml. saline only.

Table 29

The Effect of Repeated Blood Loss plus Vitamin B12 on the PPFase Activity and Cation Distribution in the Rat Erythrocytes (Average values for each group are indicated)

Date	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	p*	meq./L packed cells K^+	Na^+	Total base
Group A (0.06γ Vit. B_{12} daily)								
May 4	9.35	52.5	3	6.6		101.0	3.3	104.3
7	7.32	58.2	12	17.1	0.02	91.8	3.1	94.9
11	7.83	52.0	13	7.1	NS**	100.2	3.0	103.2
18	9.40	51.0	7	12.0	0.05	90.1	3.9	94.0
Group B (Saline only)								
May 7	9.88	53.7	2	9.0		95.0	3.3	98.3
10	6.89	53.0	9	8.3	NS	105.5	4.7	109.7
14	7.89	52.0	13	7.5	NS	103.0	3.8	106.8
21	8.90	55.0	8	9.7	NS	92.0	3.0	95.0

* Probability.
** Not significant statistically.

Rats with Walker Carcinoma 256.

Blood samples were obtained with the kind permission of Dr. A. G. Stewart from his experimental animals bearing Walker Carcinoma 256. These animals were killed three weeks after transplantation with the tumour cells. Rats No. 8, 9 and 10, were killed after four weeks.

Table 30

The Effect of Walker Carcinoma 256 on the PPFase Activity and Cation Distribution in the Rat Erythrocytes

Rat No.	Weight of rat plus tumour	Weight of tumour	Weight of spleen	RBC x 10^6	MCV (μ^3)	Retic's %	Enzyme units	meq/L packed cells		
								K^+	Na^+	Total base
1	240 gm.	35 gm.	1.8 gm.	6.00	60.0	25.	20.0	112.0	9.8	121.8
2	260	45	2.5	-	-	-	22.6	106.0	5.5	111.5
3	255	66	-	3.90	67.0	82.	28.3	112.8	7.6	120.4
4	260	62	3.2	4.10	68.4	85.	29.0	112.8	17.9	130.9
5	250	36	3.3	5.00	64.0	48.	25.0	111.0	11.9	122.9
6	255	66	3.5	-	-	-	34.4	112.0	29.0	141.0
7	250	25	1.8	6.75	78.0	19.	14.4	90.0	12.8	102.8
8	280	65	2.4	4.80	67.0	35.	15.7	99.0	8.0	107.0
9	290	100	2.7	2.85	70.0	93.	19.1	111.6	10.0	121.6
10	240	60	2.2	7.70	50.0	14.	5.0	111.5	3.1	114.6
						Average	21.3	108.0	11.5	119.5

Rats treated with p-dimethylaminoazobenzene (PDMAB).

Blood samples were obtained from animals on project "San" No. 2, at the McEacheran Cancer Research Laboratory, with the kind permission of Dr. R. B. Sandin and Dr. K. P. Kowalewski. All these animals had been treated for five months with PDMAB which is known to cause cancer of the liver. Rats No. 22 to 25, had been simultaneously treated with tetrakis-(p-dimethylaminophenyl)-1,2-dichloroethane, to test for its anti-carcinogen properties.

Table 31

The PPFase Activity and Cation Distribution in Erythrocytes of PDMAB Treated Rats

Rat No.	RBC x 10^6	MCV(μ^3)	Retic's %	Enzyme units	meq/L packed cells		
					K^+	Na^+	Total base
22				8.2	90.0	5.0	95.0
23	7.80	73	23.0	16.3	125.0	5.2	130.2
24			12.	13.3	125.0	5.4	130.4
25			20.	17.7	110.5	3.4	113.9
26				16.3	110.1	5.1	115.1
27	7.30	70	17.	15.2	107.0	3.6	120.6
28				18.2	108.0	4.4	112.4
29				14.9	113.0	4.9	117.9
31	6.50	72	20.	19.6	112.0	4.4	116.4
32			11.	20.0	106.0	5.1	111.1
			Average	16.0	110.6	4.6	116.2

Table 32

Average Values for 10 Adult Normal Rats in This Laboratory

	Mean	Range
RBC x 10^6	9.47	8.50 - 10.0
MCV(μ^3)	53.0	49.0 - 57.0
Retic's %	2.2	1.0 - 3.0
K^+ meq/L cells	99.4	92.0 - 106.0
Na^+ meq/L cells	3.0	2.5 - 4.4

4. Discussion

The preliminary observation that an increased PPFase activity was associated with an increased number of reticulocytes, has now been confirmed for both rats and rabbits. It is to be noted, however, that no quantitative relationship could be shown between the absolute number of reticulocytes and enzyme activity in the different experiments. While no attempt was made at differentiating the various "types" of reticulocytes, it is suggested that the increased enzyme activity is probably associated with the most immature forms. Koritz and Chantrenne (48) in their study of the ribonucleic acid (RNA) in reticulocytes, also emphasized the fact that it is probably the "type" of reticulocytes which determines the observed

phenomenon rather than the absolute number.

In the rabbit about 30 days was required for the enzyme activity to come back to a normal level after a single injection of AcPHZ (table 24). A peculiar phenomenon associated with anemia in the rabbits was the observed decrease in enzyme activity on the 2nd or 3rd day of every experiment. This effect cannot be ascribed to the AcPHZ as it is also evident in the experiment on blood loss (table 28).

Both groups of rats with tumours showed elevated PPFase activity (normal = 7.2±0.51 S.E.; σ = 2.2, ·: 7.2±2.2 =2.8 to 11.6).

This increased activity is believed to be due to the reticulocytosis and not due to the presence of the tumours per se. The presence of anemia and associated reticulocytosis is quite marked in the rats with Walker Carcinoma 256 and present to a lesser degree in those treated with PDMAB. Therefore it is not surprising that the greater enzyme activity is found in the former group.

Vitamin B_{12} appears to exert a statistically significant effect on enzyme activity in rats subjected to blood loss (table 29). Group A, May 7, showed an elevated activity, P = .02. The other groups showed no statistically significant difference. Also no difference in enzyme

activity in rabbits treated with vitamin B_{12} could be found (table 28). However this may be due to several factors: first, no marked anemia was present, and secondly, the dosage of vitamin B_{12} may have been too low. Bénard et al. (50) used a dosage of 15 gammas of vitamin B_{12} in their experiments with phenylhydrazine in rabbits.

The original observation made by Kerr (51) some 30 years ago, on the effect of hemorrhage on cation distribution in the erythrocytes, appears to have gone by unnoticed. He found that by repeated severe hemorrhage in the dog, K^+ increased from 6.5 to 15 mM/1000 gm. of cells, while Na^+ decreased from 104 to 93 mM/1000 gm. of cells. One of his several explanations for the observed result was a consideration of the possibility that this rise in K^+ concentration was a reflection of the presence of new corpuscles rich in K^+. Our experiments on rabbits show essentially the same results as those of Kerr's, whether the anemia was induced by AcPHZ or blood loss. That is, the final concentrations towards the end of an experiment show an elevated K^+ and decreased Na^+. Both tables 25 and 26 indicate that the gradual increase of K^+ concentration after the initial decrease, is associated with an increased number of reticulocytes. Also, the K^+ concentration is increased in rats with Walker Carcinoma

as well as in rats treated with PDMAB.

The initial reversal of the K/Na ratio observed after AcPHZ treatment (tables 25 and 26) cannot be explained at the present time. Later experiments indicated that phenylhydrazine did not affect the K^+ or Na^+ concentration in rabbit cells in vitro. A possible interpretation may be that, since circulating red blood cells are normally a mixed population of various ages, AcPHZ increases the decay process in all cells above a critical age level. The fluctuation in the Na^+ and K^+ concentration due to repeated AcPHZ injections (table 26) may be interpreted as support for this suggestion.

No definite conclusion can be drawn about the possible protective effect of vitamin B_{12} on the initial loss of K from erythrocytes in rabbits treated with AcPHZ (table 27), since only one experiment was conducted.

Today there is increasing evidence that K^+ accumulation is associated not only with carbohydrate metabolism, but also with protein synthesis and amino acid metabolism. Krebs (52) found that glutamate and K^+ were taken up in approximately equivalent amounts by brain and retina slices. Davies (53) showed that both yeast and bacteria would accumulate glutamic acid which was accompanied by an uptake of K^+. Active amino acid (C^{14} - glycine, L - histidine, L - leucine and L - lysine) uptake by

reticulocytes but not by mature cells, was demonstrated by Borsook (54), Holloway and Ripley (55) and by Koritz (48) as well as others. Also, it is now a well known fact that the ribonucleic acid (RNA) content of erythrocytes parallels the reticulocyte count (48), (57). Brachet and Caspersson (56) maintain that protein synthesis and the RNA content in cells are closely related; there is an increase of this nucleoprotein when there is increased protein synthesis. Indeed Gavosto and Rechenman (57) were able to show that the protein synthetic ability of the reticulocytes disappears almost completely with the disappearance of cytoplasmic basophilia. They claim that this supports the theory that it is the microsomes that are the direct agents of protein synthesis. Long (58) found a 60% increase in K^+ content of cancerous tissue, and concluded that increased K^+ concentration is associated with the increased mitotic frequency of the cells.

Therefore it would appear that K^+ accumulation is not associated with any one specific enzyme or metabolic process, but may be dependent on both carbohydrate and protein metabolism. However it is interesting to note that Sir Lionel Whitby (61) points out that magnesium is known to be a potent activator of the enzyme, alkaline phosphatase, that the magnesium content of the erythrocytes

is increased during active haemopoiesis, and that this increase may be associated with the presence of the enzyme (alkaline phosphatase) in the normoblastic nuclei. In a like manner, we have shown that the increase in potassium content of the erythrocytes in certain conditions parallels the increase in reticulocytes, that potassium is an activator of the enzyme pyruvic phosphoferase, and that the activity of this enzyme is greatly increased in immature cells.

Addendum

Since the writing of the above discussion, Davis, Bigelow and Alpen (83) have reported the presence of reticulocytes plus "new erythrocytes" in rats following acute hemorrhage. These "new erythrocytes" had a mean cell age of less than one week and showed statistically a highly significant increase in osmotic resistance. Therefore the presence of such "new erythrocytes" may account for the discrepancy noted in this thesis between the number of reticulocytes and the PPFase activity as discussed on page 50. It will be recalled that a very high PPFase activity was demonstrated in the immature cells of the rat, table 22 and discussed on page 37.

D. Pyruvic Phosphoferase Activity and Cation Distribution in Human Erythrocytes in Various Blood Dyscrasias.

1. Introduction

Animal experiments have indicated that in general a rise and fall in PPFase activity is associated with the reticulocyte count. Human blood disorders such as pernicious anemia (P.A.) are known to be accompanied by an intense reticulocytosis during therapy, therefore such clinical conditions should be ideal for a study of the enzyme activity in human cells.

2. Experimental

PPFase activity and reticulocyte counts were determined by the methods previously described. Cell counts were performed according to routine laboratory procedure.

Blood samples were obtained from diagnosed P.A. patients, and from all patients who had an anemia of over 50%.* All samples were analysed within 1-2 hours from the time the blood was drawn and in the special case of one patient, No. "U1", within one-half hour. Samples which were stored at the hospital in a refrigerator for 24 hours, will be indicated on the charts.

3. Results

The results obtained on the clinical patients are summarized in the following tables and a detailed discussion is given on page 67.

* Hb < 50%

Table 33

Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of P.A. cases

Case No.	Age	Sex	Enzyme Activity(U)	Retic's %	RBC x 10^6	MCV (μ^3)	meq/L packed cells K^+	meq/L packed cells Na^+	meq/L packed cells Total base	Treatment prior to obtaining sample
U1	66	M	11.0	3.5	1.11	103	89.0	10.5	99.5	Nil
U14	70	M	21.4	8.2	3.96	98	99.0	6.3	105.3	vit. B_{12} for one week
G1	97	F	36.0	18.4	1.85	92	92.2	5.6	97.8	Repeated transfusions
RA1	73	F	25.0	5.5	1.05	129	79.0	13.7	92.7	Travert, glucose prev. day
RA3	75	M	11.0	4.1	1.78	143	67.0	14.1	81.1	Liver extract, penicillin
RA7	59	F	21.6	1.9	0.89	112	66.0	31.0*	97.0	Transf. B_{12}, Dicrysticin, Thiomerin.
RA4	76	M	13.9	16.0	1.60	128	95.0	11.3	106.3	?
M3	57	F	22.0	0.3	1.00	150	-	-	-	Nil

* Samples were stored at 5°C. for 24 hrs.

Table 34

Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of Patients Diagnosed as "Anemic"

Case No.	Age	Sex	Enzyme Activity(U)	Retic's %	RBC x 10^6	MCV (μ^3)	meq/L packed cells			Diagnosis***
							K^+	Na^+	Total base	
U2	6**	M	15.7	3.2	2.35	101	93.5	4.8	98.3	Pseudohemophilia
U3	14**	M	12.6	39.0	2.72	88	81.0	10.4	91.4	Congenital hemolytic anemia
G2	40	F	19.3	15.2	1.90	92	89.5	5.4	94.9	Aleukemic myelosis
G3	27	F	6.0	3.0	2.86	86	67.3	13.0*	80.3	Uterine bleeding
RA2	5	M	5.8	0.1	3.90	87	87.0	8.2	95.2	Aplastic anemia
MI	68	F	22.5	2.0	2.80	100	83.4	12.5	96.0	Hypochromic normocytic anemia
M4	46	F	26.0	2.5	3.88	48	81.0	8.7	89.7	Nutritional anemia
M5	77	F	17.8	7.0	2.60	84	96.0	3.9	99.9	Hemorrhagic anemia

* Samples were stored at 5°C. for 24 hrs.
** Months.
*** These patients received numerous blood transfusions prior to obtaining the sample.

Table 35

Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of Patients Not Diagnosed as "Anemic"

Case No.	Age	Sex	Enzyme Activity(U)	Retic's %	RBC x 10^6	MCV (μ^3)	meq/L packed cells K^+	meq/L packed cells Na^+	meq/L packed cells Total base	Diagnosis
M2	41	F	14.5	0.8	4.80	88	74.0	14.7	88.7	Infectious hepatitis
B1	22	M	11.0	1.0	4.88		88.5	5.1	93.6	Diabetes
m2	52	M	13.0	-	3.15	94	-	-	-	Uremia
G4	49	F	14.0	2.2	3.40	75	66.0	8.7*	74.7	Uremia
m4	33	F	7.3	-	3.00	-	-	-	-	Pyelitis
M6	24	F	15.4	6.0	1.80	50	-	-	-	Acute lymphatic leukemia
M7	-	M	6.3	2.8	4.00	39	-	-	-	Acute myeloid leukemia

* Samples were stored at 5°C. for 24 hrs.

Table 36

Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of Patients with Cancer and Gastric Ulcers

Case No.	Age	Sex	Enzyme Activity(U)	Retic's %	RBC x 10^6	MCV (μ^3)	meq/L packed cells K^+	Na^+	Total base	Diagnosis
G5	51	F	28.0	3.4	3.25	68	71	15*	86	Carcinoma of ovaries with metastasis
RA5	67	F	6.9	0.5	4.58	93	90.5	8.9	99.4	Carcinoma of stomach
RA6	75	M	14.5	3.2	3.50	87	95.0	8.5	103.5	Carcinoma of stomach
RA8	-	M	4.0	-	-	-	-	-	-	Gastric ulcer
M3	81	F	12.1	-	3.36	95	-	-	-	Gastric ulcer

* Samples were stored at 5°C. for 24 hrs.

Blood from leukemic patients' was used to show the effect of leukocytes (WBC) on the red cell PPFase activity. Case No. M6 was an acute lymphatic leukemia with a white cell volume of 6% by hematocrit, whereas case No. M7 was an acute myeloid leukemia and had a white cell volume of 16% by hematocrit. To determine the PPFase activity in the absence of WBC, the buffy layer was carefully aspirated off each time the cells were washed (two times).

Table 37

Effect of Leukocytes (WBC) on Pyruvic Phosphoferase Activity in the Erythrocytes of Leukemic Patients

Case No.	Enzyme Activity (U)		Hematocrit of the WBC(%)
	Plus WBC	Minus WBC	
M6	77.0	15.4	6.0
M7	36.0	6.3	16.0

It may be concluded from Table 37 that WBC from leukemic patients possess a very high PPFase activity and that different types of WBC may possess different levels of enzyme activity.

Table 38

The Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of P.A. patients* during therapy**

Days of Treatment	nil	2	5	8	12	16	19	33
Enzyme Activity (U)	11.0	34.0	21.0	39.3	23.0	19.4	15.1	12.6
Retic's %	3.5	5.5	51.5	37.0	16.0	7.4	4.8	1.0
RBC x 10^6	1.11	1.05	1.26	1.92	2.45	3.40	3.75	4.40
MCV (μ^3)	103	105	133	110	104	91	100	82
K^+ meq/L cells	-	72.0	82.0	91.0	102.0	103.0	105.0	101.0
Na^+ meq/L cells	-	20.0	12.7	5.5	6.3	5.0	4.6	4.1
Total Base	-	92.0	94.7	96.5	108.3	108.0	109.6	105.1

* Case No. - U1
Age - 66
Sex - M
Diagnosis- P.A.

** This patient had no history of any previous treatment for P.A., and received daily injections of 30 gamma vitamin B_{12} while hospitalized.

Table 39

The Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of P.A. Patients* during therapy**

Day of Test	1	4	7
Enzyme Activity (U)	11.0	15.0	11.0
Retic's %	4.1	12.5	9.0
RBC (million)	1.78	1.81	2.38
MCV (μ^3)	143	124	111
K meq/L cells	67.0	91.3	76.3
Na meq/L cells	14.1	10.8	11.8
Total Base	81.1	102.1	88.1

* Case No. - RA3
Age - 75
Sex - M
Diagnosis - P.A.

** This patient received no vitamin B_{12}, but 15 units of liver extract up to the 7th day, and 30 units of liver extract on the 7th day.

Table 40

The Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of an Aleukemic Myelosis Patient* during therapy**

Day of Test	1	3	6	13
Enzyme Activity (U)	19.8	20.4	20.8	20.8
Retic's %	15.2	11.2	4.0	7.0
RBC (million)	1.90	2.85	2.90	3.80
MCV (μ^3)	92	80	93	93
K meq/L cells	89.5	98.0	96.0	85.0
Na meq/L cells	5.4	7.4	6.0	6.5
Total Base	94.9	105.4	102.0	91.5

* Case No. - G1
Age - 40
Sex - F
Diagnosis - Aleukemic myelosis.

** Therapy consisted of one blood transfusion a day.

Control Experiments:

For control experiments, healthy subjects in the laboratory were tested on the dates indicated in the table.

Table 41

The Effect of Time on the Pyruvic Phosphoferase Activity in the Erythrocytes of Healthy Subjects

No.	Date	Enzyme Activity (U)
B1	Dec. 3, 1953	8.3
	Apr. 29, 1954	8.0
	May 7, 1954	9.7
	May 18, 1954	8.7
B2	Dec. 3, 1953	13.1
	Jan. 26, 1954	12.6
	Feb. 5, 1954	12.1
	July 13, 1954	14.0
B3	Dec. 3, 1953	8.0
	Dec. 16, 1953	8.7
	Jan. 28, 1954	8.8
	July 20, 1954	8.5

Table 42

The Effect of Time on the Pyruvic Phosphoferase Activity and Cation Concentration in the Erythrocytes of a Healthy Subject

No.	Date	Enzyme Activity (U)	meq/L packed cells		
			K^+	Na^+	Total base
B4	July 5	14.0	89.0	9.0	98.0
	July 8	14.5	90.0	9.0	99.0
	July 13	15.2	90.5	9.3	99.8
	July 15	13.2	87.0	8.7	95.7

Table 43

The Effect of Storage at 10°C. for 24 Hours on the Cation Concentration in Normal Erythrocytes

Sample No.	Hours of Storage	meq/L cells	
		K^+	Na^+
1	0	82.5	9.1
1	24	82.0	16.8
2	0	90.0	8.7
2	24	89.0	14.0

4. Discussion

Essentially the same results were obtained as with the animal experiments: the PPFase activity and K^+ concentration increased with the reticulocyte counts (table 38). The enzyme activity was increased in most of the cases diagnosed as "anemic" (normal mean activity = 11±0.5, mean ± 2σ = 7-15), but again it is to be noted that no absolute correlation exists between the activity and reticulocyte count. For example, Case No. U3, table 34, showed a "normal" PPFase activity in the presence of 39% reticulocytes, while No. M3, table 33, had a high PPFase activity in the presence of only 0.3% reticulocytes. Five cases, G3, RA2, M7, RA8, and RA5 were below the normal range of enzyme activity and were diagnosed as uterine bleeding, aplastic anemia, acute myeloid leukemia, gastric ulcer, and carcinoma of the stomach.

Case No. RA3, table 39, is of especial interest in view of the fact that this P.A. patient showed no increase in enzyme activity during therapy yet the reticulocyte count went up to 12%. It is to be noted that this patient received liver instead of vitamin B_{12} therapy. An examination of the other tables will show that the enzyme activity generally increased in the presence of vitamin B_{12} treatment. It will be recalled that B_{12} exerted a statistically significant increase on PPFase activity in the

rats. In this connection it is of interest to note that Ling and Chow (60) suggested that vitamin B_{12} may have a role in carbohydrate metabolism.

Table 38 shows the rise and fall of enzyme activity in a P.A. patient during 30 days of therapy while table 41 indicates a ± 10% variation in activity in normal subjects over a 7 month period. PPFase activity in leukemia is indicated in table 37; the increased activity is believed to be due to the presence of WBC. Bartlett and Marlow (62) have shown that WBC have a high rate of glycolysis.

Addendum

Since the writing of this thesis, Davis, Bigelow and Alpen (83) reported the presence of "new erythrocytes" plus reticulocytes following acute hemorrhage in the rat. The "new erythrocytes" showed an increased osmotic resistance. They also quoted other workers that red cells in most anemias had an increased osmotic resistance, whereas cells in P.A. patients treated with liver therapy, the mean osmotic resistance was usually unchanged. Table 39 showed that in a P.A. patient who received liver therapy there was no increase in PPFase activity, whereas other P.A. patients on vitamin B_{12} treatment showed increased PPFase activity (table 38).

E. Summary on Phosphoferase Studies

An intracellular pyruvic phosphoferase has been shown to be present in mammalian erythrocytes. It is activated by K^+, inhibited by Na^+ or Ca^{++}, and is believed to be an SH enzyme. The activity was found to be greatly increased in immature and nucleated cells. Both the potassium content and enzyme activity were found to parallel the increase in reticulocyte count under certain conditions in man and rabbits. The activity was also found to be increased by vitamin B_{12} under certain conditions.

A study of the erythrocytes in different species indicated a higher enzyme activity for those species with a high K^+, low Na^+ content.

II. The Phospho-Glyceromutase-Enolase (PGME) System Of The Red Blood Cell

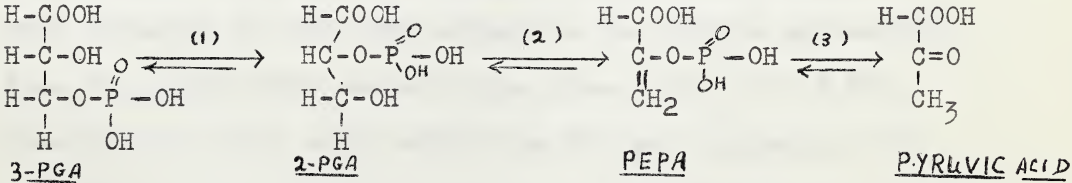
1. Introduction

Bartley (63), and Kun (64) reported the formation of phospho(enol)pyruvate (PEPA) from phosphoglyceric acid (PGA) by washed suspensions of sheep kidney particles and by tissue homogenates respectively. It has been shown previously that pyruvic phosphoferase activity occurs in the red blood cell. Therefore it was of interest to see whether the enzymes necessary for the formation of PEPA from PGA were also present in the erythrocyte.

2. Experimental

Kun (64) estimated PEPA formation from PGA by determination of the iodine labile phosphorous. However, since we have shown pyruvic phosphoferase to be present in the RBC, it was decided to utilize this highly specific form of phospho(enol)pyruvic acid estimation, by determining the liberated pyruvate.

The following equations represent the net enzymatic formation of PEPA from PGA.



The enzymes responsible for catalyzing the different steps are as follows:

Step (1) - phosphoglyceromutase

(2) - Enolase

(3) - Pyruvic phosphoferase

a. Principle of the Proposed Procedure

Kun (64) used both Mg^{++} and K^{+} ions in the incubation mixture, therefore essentially the same ionic conditions are required as that for PPFase activity. With a red cell hemolysate, any PEPA formed in such an incubation mixture could be prevented from breakdown or any further accumulation at any time by the addition of F^{-} since enolase is known to be very sensitive to F^{-} inhibition. We have already shown that PPFase is not inhibited by 10^{-2} M F^{-}. Therefore by the addition of first, F^{-}, then excess ADP, any synthesized PEPA should be acted upon by PPFase converting it to free pyruvate, which could then be determined by the dinitrophenylhydrazine method.

b. Procedure

Phosphoglyceric acid was obtained as the Ba-salt from Nutritional Biochemicals, and converted to the K-salt by treatment with HCl and K_2SO_4. Red cell hemolysates were prepared in the same manner as for PPFase estimation, i.e. the cells were washed three times with 0.15 M KCl, then diluted with water after the KCl was aspirated off.

However, it was found necessary to make a final cell dilution of 1:20 instead of the 1:50 dilution used for the PPFase determination. Human cells were used throughout the experiments except where stated otherwise.

The incubation mixture contained the following components at the final concentration indicated in a total volume of 1.0 ml. Tris buffer, 0.04 M, pH 6.8; PGA, 0.015 M; KCl, 0.10 M; $MgSO_4$, 0.005 M; KF, 0.01 M; ADP, 0.004 M.

After 5-10 minutes at 37°C. for thermoequilibrium, 0.1 ml. of hemolysate was added to 0.7 ml. of a PGA-Tris-KCl-$MgSO_4$ mixture. This was incubated for exactly 20 minutes at the end of which time 0.1 ml. of 0.1 M potassium fluoride was added. After 2 minutes, 0.10 ml. of 0.04 M ADP was added and the mixture was incubated for exactly 5 minutes. At the end of the 5 minutes, the reaction was stopped by the addition of 1.0 ml. of 0.0125% 2,4-dinitrophenylhydrazine in N HCl, incubated for 10 minutes and 3.0 ml. of 0.5 N NaOH added. After mixing, this was allowed to stand at room temperature for 5 minutes, then centrifuged for another 5 minutes at 3000 r.p.m. and the optical density of the supernatant was determined at 510 mμ in the Beckman DU spectrophotometer.

The amount of liberated pyruvate was estimated from a standard curve. Controls were run simultaneously and contained all the reagents except that after F^- addition,

0.10 ml. of water was added instead of the ADP. Such controls were used for the blank readings.

c. Kinetics

The effects of various factors on the synthesis of PEPA and therefore on the amount of pyruvate liberated, are indicated in the following tables.

Table 44

The Effect of pH on PEPA Formation

pH	P.A.μM/ml.*
6.0	0.34
6.6	0.56
6.8	0.69
7.0	0.67
7.2	0.56
8.0	0.16

* In all cases "P.A.μM/ml." will indicate the micromoles of pyruvic acid liberated from the formed PEPA in a total volume of 1.0 ml. of incubation mixture.

Table 44 indicates that the optimal pH is 6.8 which agrees with the value reported by Kun (64) for the PGME system in tissue homogenates.

The results of an experiment to determine the optimal substrate concentration, are shown in table 45.

Table 45

The Effect of PGA Concentration on PEPA Formation

PGA, M	P.A. μM/ml.
0.10	0.26
0.07	0.40
0.04	0.59
0.02	0.74
0.015	0.75
0.010	0.74
0.005	0.68

The optimal substrate concentration was found to be 0.010 - 0.020 M. (Table 45).

Table 46

The Effect of ADP Concentration on PEPA Formation

Final ADP Conc.	P.A. μM/ml.
0.008 M	0.63
0.006	0.68
0.004	0.695
0.002	0.68

Table 46 shows that the optimal ADP concentration for PEPA formation is 0.002 - 0.006 M with an optimum at 0.004 M.

It was also necessary to determine whether PEPA formation was linear with time of incubation. The results for such an experiment are shown in table 47.

Table 47

The Effect of Time of Incubation on PEPA Formation

Time	P.A. μM/ml.*
3 minutes	0.10
5	0.15
8	0.275
12	0.40
20	0.67
30	0.96

* In this case, after F^- addition, the incubation was continued for 20 minutes.

It may be concluded from the above results that PEPA formation is linear for at least 30 minutes of incubation.

Table 48

The Effect of Enzyme Concentration on PEPA Formation

Ml. Enz.	P.A. μM/ml.
0.025 ml.	0.12
0.050	0.24
0.075	0.36
0.10	0.49

Table 48 indicates that PEPA formation (up to 0.49 μM/ml.) is linear with enzyme concentration.

In the procedure outlined on page 71, it was assumed that the added fluoride would completely inhibit the enolase in the hemolysate. However it was necessary to show that such inhibition was complete under the experimental conditions used.

Table 49

Completeness of Enolase Inhibition* by Fluoride (0.01M)

Minutes	P.A. μM/ml.
2	0.69
6	0.68
12	0.68

* ADP was added at 2, 6 and 12 minutes after the F^- addition.

Thus it may be concluded that the enolase is completely inhibited by 0.01 M fluoride.

Table 50

The Effect of the Time of Incubation on the PPFase Activity

Time	P.A. μM/ml.
3 minutes	0.30
6	0.59
9	0.80
12	0.96
20	1.18

* After the ADP addition, the reaction was stopped at different times and P.A. was determined.

Under the experimental conditions outlined on page 71, the PPFase activity was found to be linear (table 50) for 6 minutes only.

d. Location of Activity in the Erythrocyte

A hemolysate was divided into two parts, one part 'A' was centrifuged at 12,000 r.p.m. for 10 min., the other 'B' was not centrifuged. Also, a concentrated ghost suspension 'C' which had been washed several times, was assayed. Table 51 indicates that the PGME system of the erythrocyte is located entirely intracellularly.

Table 51

Location of Enzyme Activity

Fraction	P.A. μM/ml.
A	0.60
B	0.56
C	nil

e. Effect of Ions

A comprehensive study of the effects of various ions on the PGME system could not be made under the experimental conditions used here. Only those ions which had either negligible or no inhibitory effect on PPFase activity could be tested on the PGME system. A detailed discussion of ion effects is reported on page 80.

Potassium.

To study the effect of K^+, the Na-salt of PGA was prepared instead of the usual K-salt and all neutralizations were carried out with NaOH. Also, the cells were washed with NaCl instead of the usual KCl procedure. After F^- addition, KCl was added for PPFase activity.

Table 52

Effect of Potassium (Human Hemolysate*)

Time	PA μM/ml.	
	In presence 0.10M KCL	In absence KCl
5 min.	0.13	0.13
12 min.	0.32	0.33
21 min.	0.56	0.56

* Human cells diluted 1:20.

It has been shown that the PPFase activity is very high in immature rat cells, therefore it was decided to try such cells as the source of the PGME enzymes.

Table 53

Effect of Potassium (Rat Hemolysate*)

Time	PA μM/ml.	
	In presence KCl	In absence KCl
5 min.	0.10	0.10
13 min.	0.24	0.24
20 min.	0.33	0.33

* Cells from day old rats, diluted 1:150

Magnesium.

After F^- addition $MgSO_4$ was added for PPFase activity.

Table 54

Effect of Mg^{++} (Rat Hemolysate*)

Time	PA μM/ml.	
	In presence of $Mg.^{++}$	In absence of $Mg.^{++}$
5 min.	0.10	nil
13 min.	0.24	nil
20 min.	0.33	nil

* Cells from day old rats diluted 1:50

f. Inhibitors

Table 55

Effect of Inhibitors*

Inhibitor	Final cone	% Inhibition
Urethane	10^{-2} M	nil
Iodoacetate	10^{-3}	nil
Dinitrophenol	10^{-3}	nil
Phenothiazine (in 50% EtOH)	10^{-3}	nil
Phenothiazone	10^{-4}	nil
ZN - insulin susp.	0.45 u total	nil
Na-arsenate	1.6×10^{-2}	36%

* Only those inhibitors which had either negligible or no inhibitory effect on PPFase activity could be tested using the procedure outlined here.

g. PGME Activity in Beef Cells

Table 56a

PGME Activity in Beef Cells*

Time	P.A. µM/ml.
5 min.	0.12
10 min.	0.24
20 min.	0.48

* Cells were diluted 1:10

4. Discussion

A rapid and specific method for the determination of PEPA has been described. It is believed that this procedure may be used in studying the species activity of the PGME system in the erythrocyte. In contrast to the PPFase enzyme, the PGME system does not require K^+ ions for maximum activity, but Mg^{++} ions are required.

It is of considerable interest to note that preliminary tests have indicated the synthesis of PEPA by beef hemolysates as Rapoport (65) claimed that there is no 2,3-diphosphoglycerate, nor the mutase necessary for its formation, in beef cells. Furthermore, it is believed (66) that the 2,3-diphosphoglycerate functions as a catalyst in the conversion of 3-PGA to 2-PGA which is the immediate precursor of PEPA. Rapoport and Guest (67) also showed that there is no 2,3-diphosphoglycerate in avian

erythrocytes; however no chick blood was available in our laboratory at the time to test the erythrocytes for any PEPA synthesis from PGA.

The PGME system was shown to be entirely intracellular with no activity in the ghosts.

III. The Choline Acetylase-Cholinesterase System of the Erythrocyte

A. Choline Acetylase Activity of the Erythrocyte

1. Introduction

It has already been indicated (p. 12) that choline acetylase is a K^+ activated enzyme. Greig (28h) reported that extracts of acetone powders of human erythrocytes could synthesize acetylcholine, therefore it was desirable to study the choline acetylase activity and its relation to potassium concentration in the erythrocyte under conditions similar to those for the PPFase study. Nachmansohn (37) also reported an active choline acetylase preparation, not from red cells but from rat brain acetone powders.

2. Experimental

Choline acetylase preparations were made from acetone powders of red cells according to the procedure used by Greig (28h).

Fresh human erythrocytes were washed three times with 1.0% saline prior to the addition to the acetone. Rats were killed by decapitation and the brains removed within 2 minutes into the cold acetone.

Acetone powder of the rat brains or human erythrocytes was prepared as follows. The material was ground in 10 volumes of cold (-10°C.) acetone (redistilled) for 15-20 minutes, filtered on a Buchner funnel, air dried, and kept over $CaCl_2$ in the freezer compartment of a refrigerator for a maximum of two days.

For extracting the powder a solution of the following

composition was used: KCl, 0.10 M; Cysteine, 0.10 M; $MgSO_4$, 0.005 M; Tris buffer, 0.02 M, pH 7.2. The acetone powders were ground in a cold room at 5°C. for 15 minutes then allowed to stand for another 15 minutes. The extractions were made in the following proportions: erythrocyte powder, 300 mg./ml., rat brain powder, 100 mg./ml. The suspension was centrifuged at 3000 r.p.m. for 15 minutes. Part of the supernatant was used for enzyme activity determinations and a portion was boiled to inactivate the enzyme for the control experiments.

Other additions and their final concentrations were as follows*: NH_2OH, 0.05 M; K acetate, 0.05 M; KCl, 0.08 M; Tris buffer, 0.02 M, pH 7.2; ATP, 0.005 M; CoA (Krishell), 2 mg./ml.; enzyme preparation 0.2 ml./ml.; cysteine, 0.02 M. The mixture was incubated at 37°C.

Acethydroxamic acid was measured in 1.0 ml. aliquots of the incubation mixture. The samples were brought to pH 1.0-1.4 with 2.0 ml. of HCl-TCA mixture, 2.0 ml. of 5% ferric chloride was added, the mixture was filtered and the absorption of the filtrate was read at 540μm in a Beckman DU quartz spectrophotometer. Micro-moles of acethydroxamic acid were estimated from a standard acetylcholine curve (acetylcholine is converted stoichiometrically to hydroxamic acid according to the procedure of Hestrin (68).

* The following list of abbreviations will be used: Tris, tris(hydroxymethyl)aminomethane; CoA, coenzyme A; ATP = adenosinetriphosphate; Ach., acetylcholine; AHA, acethydroxamic acid; TCA, trichloroacetic acid.

3. Results

Six different experiments with erythrocyte extracts were carried out, with larger concentrations of, ATP, enzyme preparation, etc.; however all experiments gave negative results in the case of red cell powders. Typical results are recorded in the following tables.

Table 56b

Acethydroxamic Acid Synthesis by Aqueous Extracts of Acetone Powders of Rat Brains, Human Erythrocytes, and "Ghosts"

Minutes After ATP Addition	Acethydroxamic acid, μM/ml.		
	Brain	Red cell	Ghost
30	0.10	0	0
60	0.20	0	0
240	0.84	0	0

Initial experiments following Greig's (28h) procedure of using choline and eserine in the incubation mixture indicated some acetylcholine formation as estimated by Hestrin's method (68). However, the blanks were found to be very high, and upon further investigation it was found that this color with a maximum absorption peak at 485 mμ was due to eserine. Indeed this is the procedure for the quantitative estimation of eserine,

that is, eserine in alkali forms rubroeserine with an absorption maximum at 485 mμ(67). Greig herself reported that Hestrin's method as compared to the bioassay procedure, gave up to 6 times the latter value, however she failed to mention that this was due to eserine. Since hydroxylamine acylation is known to be irreversible (69) there was no necessity for a cholinesterase inhibitor in the presence of hydroxylamine, and therefore because of the irreversibility of the reaction, this method should be superior to that used by Greig.

Table 57

Exerine* interference with acetylcholine estimation**

Ach. μM/ml.	
Plus eserine	Minus eserine
3.25	0

* 0.002 M
** Hestrin's method.

4. Discussion

No acethydroxamic acid formation by human erythrocyte extracts could be detected under identical conditions which showed its formation by rat brain powder extracts.

Recently Sheppard and Matthias (29) also failed to confirm Greig's findings. It is of interest to note that Greig participated as a consultant in Sheppard's (29) work. Since it has been shown that pyruvic phosphoferase activity is greatly increased in immature rat cells, it would be of interest to repeat the above experiments with immature rat cells as the source of enzyme.

B. Red Cell Choline Esterase and Acetylcholine Synthesis

1. Introduction

The reversal of acetylcholine hydrolysis by purified acetylcholine esterase (ACHE) from the eel Electrophorus electricus, has been shown by Hestrin (69) to be a function of pH. Since no acethydroxamic acid synthesis by the choline acetylase of the red cell could be demonstrated, it was decided to try Hestrin's procedure and see whether the red cell choline esterase could form acethydroxamic acid.

2. Experimental

Red cells from the Red Cross* were used as a source of ACHE. The stability of cholinesterase was demonstrated by Keilin (70) who found 100% activity in blood samples which had been stored for 40 years. The cells were washed three times with 1% saline and hemolysed with ten volumes of distilled water at 8°C. for 12 hours. It was found that by using 10 volumes of water, the ghosts would settle out without acidification. The water was decanted and the ghosts were diluted to the original volume with water. As soon as the ghosts settled out, the process was repeated until the supernatant was only a light pink in color. This required a total 4-5 washings. The ghosts were then centrifuged at 3000 r.p.m. for 15

* Blood Donor Clinic

minutes, diluted 1:5 with water, and kept as "dilute stock enzyme" in the frozen state.

The substrate was prepared fresh each time immediately before use, and consisted of Na acetate, 1.0 M, pH 6.3, and NH_2OH, 1.0 M. The incubation mixture consisted of 2.0 ml. of substrate, and 1.0 ml. of "dilute stock enzyme". This was incubated at 25°C. Control experiments contained boiled enzyme.

Acethydroxamic acid was estimated on 1.0 ml. of incubation mixture by the procedure previously outlined (p. 83).

3. Results

The results are summarized in the following tables and a detailed discussion presented on page 94.

Table 58

Preliminary Results*

Notes	AHA µM/ml.
Standard procedure	2.12
No enzyme added	0
In presence of 0.5 M choline	0.56

* Incubation time was 2 hours, and each experiment was in duplicate.

Table 59

Ethyl Acetate Compared With Sodium Acetate as Substrate

Substrate	Concentration(M)*	AHA μM/ml.
NaAc	0.70 M	0.42
EtAc	0.14 M	6.0

* The hydroxylamine was 0.70 M in both cases, and the incubation time was two and a half hours with the "dilute stock enzyme" diluted 1:4.

Table 60

Effect of Enzyme Concentration on AHA Synthesis*

Ml. Enzyme	AHA μM/ml.
0.25 ml.	0.13
0.50	0.25
0.75	0.37
1.0	0.50

* Incubation time, 2 hours, "dilute stock enzyme" diluted 1:4; NaAc and NH_2OH, 0.7 M each.

Table 61

Effect of Enzyme Concentration on AHA Synthesis in Presence of Ethyl Acetate*

Ml. enzyme	AHA μM/ml.		
	15 minutes	30 minutes	60 minutes
0.25	0.11	0.23	0.44
0.50	0.23	0.47	0.94
1.0	0.46	0.90	1.81

* Ethyl acetate 0.14 M, NH_2OH 0.7 M, Tris buffer pH 6.3, with a new enzyme preparation.

Table 62

Effect of Hydroxylamine Concentration on AHA Synthesis*

(NH_2OH)	AHA μM/ml.				
	0.5 hour	1 hour	2 hours	3 hours	5 hours
0.70 M	-	-	0.50	0.70	-
0.50	-	0.18	0.35	0.54	0.92
0.25	0.06	0.09	0.18	0.26	0.47
0.10	0.01	0.03	0.05	0.10	0.16
0.05	0.00	0.00	0.00	0.01	0.04

* NaAc, 0.70 M, "dilute stock enzyme" diluted 1:4.

Table 63

Effect of Choline on AHA Synthesis*

Incubation time in hours	AHA μM/ml.	
	plus choline	minus choline
0.5	0.06	0.18
1	0.13	0.36
3	0.42	1.06
5	0.63	1.26

* Both NaAc and NH_2OH, 0.5 M each, choline, 0.5 M, control, 0.5 M NaCl in place of choline.

Table 64

Effect of Choline Esterase Inhibitors and SH Inactivators on AHA Synthesis*

Inhibitor	Conc. (M)	AHA μM/ml.		
		15 min.	30 min.	60 min.
Control	-	0.09	0.18	0.36
$CuSO_4$	10^{-3}M	0.09	0.18	0.36
PCMB**	10^{-3}	0.09	0.18	0.36
DFP[3]	10^{-4}	0.04	0.09	0.18
Eserine	1.54×10^{-5}	0.00	0.01	0.01

* Ethyl acetate, 0.2 M, NH_2OH, 0.7 M, Tris, 0.25 M, pH 6.3.

** PCMB = p-chloromercuribenzoate.

[3] DFP = di-isopropylfluorophosphate.

Table 65

Effect of Cations on AHA Synthesis*

Cation	Conc. (M)	AHA μM/ml.		
		15 min.	30 min.	60 min.
Control	-	0.10	0.20	0.41
K^+	10^{-2}	0.10	0.20	0.41
Na^+	10^{-2}	0.10	0.20	0.41
NHy^+	10^{-2}	0.10	0.20	0.41
Mg^{++}	10^{-2}	0.10	0.20	0.41
Ca^{++}	10^{-2}	0.10	0.20	0.41

* Ethyl acetate, 0.2 M, NH_2OH, 0.7 M, Tris, 0.25 M, pH 6.3.

To test the effect of pH on acetylcholine synthesis and hydrolysis, the following substrates were used: for synthesis, NaAC, and choline at 0.70 M each, with the control containing 0.7 M NaCl in place of choline; for hydrolysis, NaCl, 0.7 M, and acetylcholine added at 30 μM/ml. of final incubation mixture, Tris buffer, 0.25 M.

Table 66

Effect of pH on Acetylcholine Synthesis and Hydrolysis*

pH	Acetylcholine, μM/ml.									
	Synthesis					Hydrolysis (Ach. remaining)				
	Time in Hours					Time in Hours				
	0.5	1	2	3	4	0.5	1	2	3	4
5.1	0.08	0.09	0.22	0.25	0.35	22.05	14.30	4.70	1.13	0.40
5.9	0.02	0.06	0.13	0.15	0.17	12.0	1.63	0.26	0.22	0.17
7.0	0.01	0.02	0.06	0.07	0.15	6.65	0.33	0.22	0.22	0.17

* Non enzymatic hydrolysis under these conditions was not detectable.

4. Discussion

Red cell acetyl cholinesterase has been shown capable of synthesizing acethydroxamic acid and acetylcholine by a reversal of its usual hydrolytic process. Essentially all of Hestrin's observations (69) on factors affecting the AHA synthesis have been confirmed with an enzyme preparation from the erythrocytes. An acid shift of pH was found to favor synthesis of Ach. (table 66).

Wilson, Bergmann and Nachmansohn (74) from a study of the active surface of ACHE, predicted that formation of AHA and Ach. should be favored by an ester substrate, and showed this to be true experimentally. Table 59 indicates the effect of ethyl acetate as compared to sodium acetate with ACHE from erythrocytes.

The inhibitory effect of choline (table 63) is explained by Hestrin as being due its ability to combine with the enzyme at an active site. Hestrin also discusses the possibility of cholinesterase supplementing choline acetylase synthesis of acetylcholine *in vivo*. However he points out that the amount of acetylcholine at equilibrium is minute in comparison with the concentration of the other participants of the system.

In the experimental conditions used, no cation effects at 10^{-2} M could be found (table 65). Mounter and Whittaker (71) from their studies on cholinesterase concluded

"there is no evidence that cholinesterase requires metal ions in the active center". Furthermore, (table 64) no inhibition due to SH group inhibitors could be detected, which agrees with the findings of Markwardt (72) and Mounter and Whittaker (71).

Hydroxamic acid formation was found to be inhibited by choline, eserine and DFP, all well known ACHE inhibitors (table 64).

C. Summary

Attempts to demonstrate acetylcholine synthesis by red cell powder extracts were negative under identical conditions which showed presence of activity from brain extracts.

Hestrin showed the synthesis of acetylcholine by purified acetylcholine esterase from the electric eel. Using his methods we were able to duplicate his results with an acetyl cholinesterase preparation from the erythrocytes.

We have shown that an acid pH favors acetylcholine synthesis, by the ACHE of erythrocytes and there is reason to believe that the H^+ concentration may be significantly higher at the membrane interface without a concomittant increase in the surrounding medium (75).

The occurrence of free choline (1-2γ/ml.) in the

plasma has been shown by Bligh (76). Furthermore, it is well known that the ACHE of the erythrocyte is found in the membrane. Hirsch (77) has demonstrated that Ach. is present mainly on the surface of the red blood cell and therefore he claims that this explains the absence of Ach. activity in native blood when tested pharmacologically. However to what extent such a mechanism may contribute to the physiological synthesis of acetylcholine by the erythrocyte it is difficult to say at this time.

IV. Nucleotide Synthesis By Human Erythrocytes And The Effect On Cation Concentration

1. Introduction

Leder and Handler (73) reported the synthesis and identification of nicotinamide mononucleotide (NMN) in human erythrocytes *in vitro*. They* pointed out that previous workers in similar experiments identified the synthesized material as diphosphopyridine nucleotide (DPN), therefore in the following experiments the synthesized material will be referred to simply as "nucleotide" as no attempts were made at differentiating between the NMN and DPN. They also showed that the material synthesized was not freely permeable to the erythrocyte membrane. It is known that a proton transfer is involved in the reduction of DPN^+,

$$DPN^+ + 2e^- + H^+ \longrightarrow DPNH$$

therefore it was of interest to see whether there was any effect on the cation content of the erythrocyte during nucleotide synthesis.

2. Experimental

The synthesized nucleotides were determined by the fluorometric procedure of Leder and Handler (73).

Fresh human cells were washed four times at $5^{\circ}C$. with four volumes of Ringer-phosphate pH 7.2, containing

*Leder and Handler

250 mg. percent glucose. After the last centrifugation, the cells were resuspended in fresh Ringer-phosphate to a hematocrit of approximately 50 percent. To each ml. of cell suspension taken for incubation, 0.1 ml. of a 22 percent nicotinamide solution was added to give a final concentration of 2 percent nicotinamide.

This served both as a substrate for the synthesis and as an inhibitor of the DPNase of the cell. Penicillin at 40 units per ml. of incubation mixture completely inhibited bacterial growth. The absence of contamination was checked by the Department of Bacteriology. The cell suspensions were incubated at 37°C. under 100 percent oxygen. Leder and Handler showed that nucleotide synthesis was inhibited by 0.01 M fluoride, therefore two control experiments were set up with each experiment on nucleotide synthesis from nicotinamide; one control contained no nicotinamide, while the second control contained nicotinamide in the presence of 0.01 M fluoride.

Results are reported as micromoles of nucleotide per ml. of packed erythrocytes.

The procedure for sodium and potassium analysis has been previously outlined (p. 39).

The intracellular pH was determined by centrifuging 1.0 ml. aliquot of the incubation mixture. The cells were then washed once with 12 ml. of 0.3 M sucrose,

centrifuged, and hemolysed with 2.0 ml. of distilled water plus saponin, and the pH determined with a Beckman glass electrode.

3. Results

Five experiments were made, all of which yielded essentially the same data; therefore only the results for one experiment will be shown in the following table.

Table 67

Effect of Nucleotide Synthesis on the Cation Concentration in the Human Erythrocyte

Time of Incubation (Hours)	0	1	3	5	9	12	18	21	24
In Presence of Nicotinamide									
Nucleotide* (μM/ml.)	0.11	0.18	0.28	0.43	0.78	1.0	1.40	1.42	1.41
K^+ meq/L** cells	81.5	76.0	69.0	66.0	64.0	62.0	57.0	56.0	54.0
Na^+ meq/L cells	6.0	6.0	6.0	6.0	6.0	5.5	5.0	5.0	5.0
pH (intracellular)	7.19	7.17		6.94	6.87	6.77			6.63
In Absence of Nicotinamide									
Nucleotide (μM/ml.)	0.11	0.13	0.14	0.14	0.11	0.13	0.10	0.13	0.14
K^+ meq/L cells	81.0	81.0		80.0	82.0		83.0		83.0
Na^+ meq/L cells	6.0	5.5	6.0	6.0	6.0	5.5	5.5	5.0	5.0
pH (intracellular)	7.18	7.18		6.97	6.82	6.72			6.57
In Presence of Nicotinamide plus Fluoride									
Nucleotide (μM/ml.)	0.11	0.14	0.14	0.13	0.11	0.13	0.11	0.12	0.14
K^+ meq/L cells	81.0	62.0	40.0	28.0	26.0	24.0	22.0	20.0	18.0
Na^+ meq/L cells	6.0	6.0	9.0	10.0	11.5	11.5	14.0	14.5	14.5
pH (intracellular)	7.21	7.25		7.25	7.21	7.21			7.25

* Micromoles of nucleotide per ml. of packed cells.

** Milli-equivalents per liter of packed cells.

4. Discussion

There was no evidence for accumulation of potassium in the presence of nucleotide synthesis by the human erythrocytes. The observed loss of potassium from erythrocytes actively synthesizing nucleotides may be due to potassium leakage which resulted from the slight amount of hemolysis noted to be present. Sodium and pH changes parallel those in the control which had no nicotinamide. The loss of potassium and gain in sodium in the presence of fluoride has been observed by many workers (79), (80), (81), and has been attributed to the inhibition of glycolysis.

FLAME PHOTOMETRY

1. Introduction

The sample containing the element to be analysed is atomized into a hot flame which acts as the light source for the spectrophotometer. The flame excites atoms in the sample causing them to emit radiations at different wavelengths which are characteristic for that element. Therefore, by selecting the strongest emission line, quantitative determinations can be made by the use of a "standard".

2. Procedure

Sodium and potassium determinations were carried out in a Beckman D U spectrophotometer with a photomultiplier attachment (78) and with an acetylene atomizer burner, No. 4030.

Instructions for operating the atomizer burner are essentially those contained in the Beckman bulletin No. 259. Oxygen should always be turned on first, and off last. The Oxygen tank valve is adjusted to 20 psi*, then the acetylene tank valve to 5 psi. With a lighted match held just below the burner tip the oxygen valve on the Beckman ("air regulator valve") is adjusted to 5 psi, then the acetylene valve is slowly opened (not over 1 psi) until the burner starts. Only the oxygen ("air regulator valve") valve is used on the panel, the other oxygen valve being left open.

*pounds per square inch.

Once the burner is going, the oxygen valve on the panel is turned up to 12 psi, and the acetylene valve to indicate 2 1/2 psi. Such settings are purely arbitrary and will vary from day to day. The exact settings appear to be dependent on the condition of the burner or, in other words, it is the appearance of the flame that determines the settings. The cone of the flame should be 1 - 1 1/2 inches high, blue, and symmetrical. If the cone is jagged or streaks of yellow showing, the acetylene is turned off and the burner cleaned with the oxygen left on. Only a finger nail can be used to clean the salt incrustation off the burner tip as any hard material would damage the capillary. A sulfuric acid cleaning solution should not be used as there is danger of damaging the capillary. Ortho-dichlorobenzene has been found useful as a cleaning agent, however it should not be atomized through the burner with the flame on as it is explosive besides being very toxic.

3. Experimental

a standard curves.

Stock standard solutions were made up as follows: for K^+, 3.728 gm. of KCl (1.955 gm. K^+ /L) was made up to a liter with distilled water, which is equal to 50.0 meq. of K /L; for Na^+, 2.923 gm. of NaCl (1.150 gm. Na^+ /L) was made up to a liter of distilled water, which is equal to 50.0 meq. of Na^+ /L; for Ca^{++}, 439.7 mg. of Ca $(C_2H_3O_2)_2.H_2O$

(10.0 mg. Ca^{++}/100 ml.) was made up to a liter of distilled water, which is equal to 5.0 meq. of Ca^{++}/L. These stock standards were kept in siliconed bottles and "working standards" were checked against commercial flame standards distributed by the College of American Pathologists.

"Working standards" were prepared to contain all the ions, and at the approximate concentrations as in the unknown samples. Such standards were also diluted in the same proportion as the unknowns. When doing determinations the meter needle of the photomultiplier unit was set at 50 percent transmittance with the particular standard being used. This setting was checked with the standard immediately before and after each unknown reading, in this way eliminating any errors in readings due to fluctuations in gas pressure, or clogging of the burner.

Data for standard curves are summarized in the following tables.

(i) Serum Standard Curves.

Working standards were prepared to contain 150.0 meq/L of Na^{+} and 5.0 meq/L of K^{+}, diluted 1:50. The serum was also diluted 1:50. Background readings were taken with

distilled water. The serum sodium concentration was then determined from a standard curve. Potassium readings are linear with concentration and therefore the serum potassium can be calculated directly from the unknown reading.

Table 68

Serum sodium* Standard Curve

Na^+ meq/L std. sol'n	%T
0.0	0.0
5.0	4.0
25.0	17.0
50.0	28.0
75.0	35.0
100.0	41.0
125.0	46.0
150.0	50.0
175.0	54.0

*The readings were made with the solution containing 150.0 meq/L of Na^+, as the standard solution set at 50.0%T. All readings were made in duplicate with the standard being read immediately before and after each reading. Solutions containing K^+ and Ca^{++} at 5.0 meq/L gave identical readings, i.e. Ca^{++} and K^+ at 5.0 meq/L, diluted 1:50 have no effect on serum Na^+ determinations. The

589 mμ sodium emission line was used.

Table 69

Serum Potassium Standard Curve

K^+	%T (minus background)	
meq/L	K^+ only	K^+ plus Na^+ 150.0 meq/L
2.5	23.0	25.0
5.0*	44.0	50.0
7.5	67.0	75.0
10.0	89.0	96.0

*This solution was used as the standard and was set at 45.0 %T. The 765 mμ potassium emission line was used.

(ii) Erythrocyte Standard Curves.

Working standards were prepared to contain 80.0 meq/L of K^+ and 10.0 meq/L of Na^+ diluted 1:200. Red cell hemolysates were diluted on the basis of 1 ml. of packed cells to 200 ml. with water. The exact concentrations were then calculated on the basis of micro-hematocrit valves. Data for standard curves are shown in the following tables.

Table 70

Erythrocyte Sodium Standard Curve

Na^+ meq/L	%T (minus background) Na^+ only	Na^+ plus K^+ 150 meq/L
2.0	7.0	7.5
5.0	17.0	18.0
10.0	34.0	35.5
15.0	50.0	55.0
20.0	64.0	67.0

Table 71

Erythrocyte Potassium Standard Curve

K^+ meq/L	%T
10.0	6.5
25.0	16.0
50.0	33.0
75.0	49.0
100.0	65.0

(iii) Reproducibility of determinations on erythrocytes. Table 72 shows duplicate results of three different blood samples. The procedure for erythrocyte cation determination has been described on p. 39.

Table 72

Reproducibility of Sodium and Potassium Determinations in Erythrocytes

Sample No.	K^+		Na^+	
	% T	meq/l	% T	meq/l
1a	50.0	80.0	44.5	9.1
1b	50.0	80.0	44.5	9.1
2a	54.0	86.4	44.0	9.0
2b	54.0	86.4	44.0	9.0
3a	51.5	82.4	64.0	13.8
3b	51.5	82.4	64.5	13.9

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